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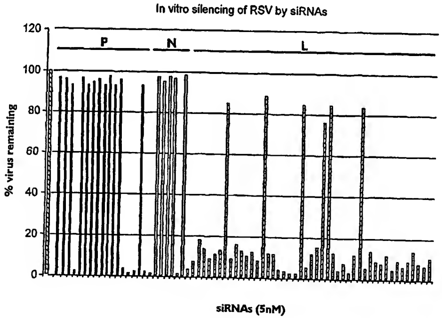

(57) Abstract: The present invention is based on the in vivo demonstration that RSV can be inhibited through intranasal administration of iRNA agents as well as by parenteral administration of such agents. Further, it is shown that effective viral reduction can be achieved with more than one virus being treated concurrently. Based on these findings, the present invention provides general and specific compositions and methods that are useful in reducing RSV mRNA levels, RSV protein levels and viral titers in a subject, e.g., a mammal, such as a human. These findings can be applied to other respiratory viruses.

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RNAi MODULATION OF RSV AND THERAPEUTIC USES THEREOF

RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Application No. 60/642,364, filed January 7, 2005, which is incorporated herein by reference in its entirety.

TECHNICAL FIELD

The invention relates to the field of respiratory syncytial viral (RSV) therapy and compositions and methods for modulating viral replication, and more particularly to the down-regulation of a gene(s) of a respiratory syncytial virus by oligonucleotides via RNA interference which are administered locally to the lungs and nasal passage via inhalation/intranasally or systemically via injection/intravenous.

BACKGROUND

By virtue of its natural function the respiratory tract is exposed to a slew of airborne pathogens that cause a variety of respiratory ailments. Viral infection of the respiratory tract is the most common cause of infantile hospitalization in the developed world with an estimated 91,000 annual admissions in the US at a cost of $300 M. Human respiratory syncytial virus (RSV) and parainfluenza virus (PIV) are two major agents of respiratory illness; together, they infect the upper and lower respiratory tracts, leading to croup, pneumonia and bronchiolitis (Openshaw, P.J.M.. Respir. Res. 3 (Suppl 1), S15-S20 (2002), Easton, A.J., et al., *Clin. Microbiol. Rev.* 17, 390-412 (2004)). RSV alone infects up to 65% of all babies within the first year of life, and essentially all within the first 2 years. It is a significant cause of morbidity and mortality in the elderly as well. Immunity after RSV infection is neither complete nor lasting, and therefore, repeated infections occur in all age groups. Infants experiencing RSV bronchiolitis are more likely to develop wheezing and asthma later in life. Research for effective treatment and vaccine against RSV has been ongoing for nearly four decades with few successes (Openshaw, P.J.M.. Respir. Res. 3 (Suppl 1), S15-S20 (2002), Maggon, K. et al, *Rev. Med. Virol.*

14, 149-168 (2004)). Currently, no vaccine is clinically approved for either RSV. Strains of both viruses also exist for nonhuman animals such as the cattle, goat, pig and sheep, causing loss to agriculture and the dairy and meat industry (Easton, A.J., et al., *Clin. Microbiol. Rev.* **17**, 390-412 (2004)).

Both RSV contain nonsegmented negative-strand RNA genomes and belong to the *Paramyxoviridae* family. A number of features of these viruses have contributed to the difficulties of prevention and therapy. The viral genomes mutate at a high rate due to the lack of a replicational proof-reading mechanism of the RNA genomes, presenting a significant challenge in designing a reliable vaccine or antiviral (Sullender, W.M. *Clin. Microbiol. Rev.* **13**, 1-15 (2000)). Promising inhibitors of the RSV fusion protein (F) were abandoned partly because the virus developed resistant mutations that were mapped to the F gene (Razinkov, V., et. al., *Antivir. Res.* **55**, 189-200 (2002), Morton, C.J. *et al. Virology* **311**, 275-288 (2003)). Both viruses associate with cellular proteins, adding to the difficulty of obtaining cell-free viral material for vaccination (Burke, E., et al., *Virology* **252**, 137-148 (1998), Burke, E., et al., *J. Virol.* **74**, 669-675 (2000), Gupta, S., et al., *J. Virol.* **72**, 2655-2662 (1998)). Finally, the immunology of both, and especially that of RSV, is exquisitely complex (Peebles, R.S., Jr., et al., *Viral. Immunol.* **16**, 25-34 (2003), Haynes, L.M., et al., *J. Virol.* **77**, 9831-9844 (2003)). Use of denatured RSV proteins as vaccines leads to "immunopotentiation" or vaccine-enhanced disease (Polack, F.P. *et al. J. Exp. Med.* **196**, 859-865 (2002)). The overall problem is underscored by the recent closure of a number of anti-RSV biopharma programs.

The RSV genome comprises a single strand of negative sense RNA that is 15,222 nucleotides in length and yields eleven major proteins. (Falsey, A. R., and E. E. Walsh, 2000, Clinical Microbiological Reviews 13:371-84.) Two of these proteins, the F (fusion) and G (attachment) glycoproteins, are the major surface proteins and the most important for inducing protective immunity. The SH (small hydrophobic) protein, the M (matrix) protein, and the M2 (22 kDa) protein are associated with the viral envelope but do not induce a protective immune response. The N (major nucleocapsid associated protein), P (phosphoprotein), and L (major polymerase protein) proteins are found associated with virion RNA. The two non-structural

proteins, NS1 and NS2, presumably participate in host-virus interaction but are not present in infectious virions.

Human RSV strains have been classified into two major groups, A and B. The G glycoprotein has been shown to be the most divergent among RSV proteins. Variability of the RSV G glycoprotein between and within the two RSV groups is believed to be important to the ability of RSV to cause yearly outbreaks of disease. The G glycoprotein comprises 289-299 amino acids (depending on RSV strain), and has an intracellular, transmembrane, and highly glycosylated stalk structure of 90 kDa, as well as heparin-binding domains. The glycoprotein exists in secreted and membrane-bound forms.

Successful methods of treating RSV infection are currently unavailable (Maggon K and S. Barik, 2004, Reviews in Medical Virology 14:149-68). Infection of the lower respiratory tract with RSV is a self-limiting condition in most cases. No definitive guidelines or criteria exist on how to treat or when to admit or discharge infants and children with the disease. Hypoxia, which can occur in association with RSV infection, can be treated with oxygen via a nasal cannula. Mechanical ventilation for children with respiratory failure, shock, or recurrent apnea can lower mortality. Some physicians prescribe steroids. However, several studies have shown that steroid therapy does not affect the clinical course of infants and children admitted to the hospital with bronchiolitis. Thus corticosteroids, alone or in combination with bronchodilators, may be useless in the management of bronchiolitis in otherwise healthy unventilated patients. In infants and children with underlying cardiopulmonary diseases, such as bronchopulmonary dysphasia and asthma, steroids have also been used.

Ribavirin, a guanosine analogue with antiviral activity, has been used to treat infants and children with RSV bronchiolitis since the mid 1980s, but many studies evaluating its use have shown conflicting results. In most centers, the use of ribavirin is now restricted to immunocompromised patients and to those who are severely ill.

The severity of RSV bronchiolitis has been associated with low serum retinol concentrations, but trials in hospitalized children with RSV bronchiolitis have shown that vitamin A supplementation provides no beneficial effect. Therapeutic trials of 1500 mg/kg

intravenous RSV immune globulin or 100 mg/kg inhaled immune globulin for RSV lower-respiratory-tract infection have also failed to show substantial beneficial effects.

In developed countries, the treatment of RSV lower-respiratory-tract infection is generally limited to symptomatic therapy. Antiviral therapy is usually limited to life-threatening situations due to its high cost and to the lack of consensus on efficacy. In developing countries, oxygen is the main therapy (when available), and the only way to lower mortality is through prevention.

RNA interference or "RNAi" is a term initially coined by Fire and co-workers to describe the observation that double-stranded RNA (dsRNA) can block gene expression when it is introduced into worms (Fire *et al.*, *Nature* 391:806-811, 1998). Short dsRNA directs gene-specific, post-transcriptional silencing in many organisms, including vertebrates, and has provided a new tool for studying gene function. RNAi has been suggested as a method of developing a new class of therapeutic agents. However, to date, these have remained mostly as suggestions with no demonstrate proof that RNAi can be used therapeutically.

Therefore, there is a need for safe and effective vaccines against RSV, especially for infants and children. There is also a need for therapeutic agents and methods for treating RSV infection at all ages and in immuno-compromised individuals. There is also a need for scientific methods to characterize the protective immune response to RSV so that the pathogenesis of the disease can be studied, and screening for therapeutic agents and vaccines can be facilitated. The present invention overcomes previous shortcomings in the art by providing methods and compositions effective for modulating or preventing RSV infection. Specifically, the present invention advances the art by providing iRNA agents that have been shown to reduce RSV levels in vitro and in vivo, as well as being effective against both major subtypes of RSV, and a showing of therapeutic activity of this class of molecules.

SUMMARY

The present invention is based on the *in vitro and in vivo* demonstration that RSV can be inhibited through intranasal administration of iRNA agents, as well as by parenteral

administration of such agents, and the identification of potent iRNA agents from the P, N and L gene of RSV that can reduce RNA levels with both the A and B subtype of RSV. Based on these findings, the present invention provides specific compositions and methods that are useful in reducing RSV mRNA levels, RSV protein levels and RSV viral titers in a subject, *e.g.*, a mammal, such as a human.

The present invention specifically provides iRNA agents consisting of, consisting essentially of or comprising at least 15 or more contiguous nucleotides of one of the genes of RSV, particularly the P, N and L genes of RSV, and more particularly agents that comprising 15 or more contiguous nucleotides from one of the sequence provided in Table 1 (a-c). The iRNA agent preferably consists of less than 30 nucleotides per strand, *e.g.*, 21-23 nucleotides, such as those provided in Tables 1 (a-c). The double stranded iRNA agent can either have blunt ends or more preferably have overhangs of 1-4 nucleotides from one or both 3' ends of the agent.

Further, the iRNA agent can either contain only naturally occurring ribonucleotide subunits, or can be synthesized so as to contain one or more modifications to the sugar or base of one or more of the ribonucleotide subunits that is included in the agent. The iRNA agent can be further modified so as to be attached to a ligand that is selected to improve stability, distribution or cellular uptake of the agent, e.g. cholesterol. The iRNA agents can further be in isolated form or can be part of a pharmaceutical composition used for the methods described herein, particularly as a pharmaceutical composition formulated for delivery to the lungs or nasal passage or formulated for parental administration. The pharmaceutical compositions can contain one or more iRNA agents, and in some embodiments, will contain two or more iRNA agents, each one directed to a different seqment of a RSV gene or to two different RSV genes.

The present invention further provides methods for reducing the level of RSV viral mRNA in a cell. Such methods comprise the step of administering one of the iRNA agents of the present invention to a subject as further described below. The present methods utilize the cellular mechanisms involved in RNA interference to selectively degrade the viral mRNA in a cell and are comprised of the step of contacting a cell with one of the antiviral iRNA agents of the present invention. Such methods can be performed directly on a cell or can be performed on a

mammalian subject by administering to a subject one of the iRNA agents/pharmaceutical compositions of the present invention. Reduction of viral mRNA in a cells results in a reduction in the amount of viral protein produced, and in an organism, results in a decrease in replicating viral titer (as shown in the Examples).

The methods and compositions of the invention, e.g., the methods and iRNA agent compositions can be used with any dosage and/or formulation described herein, as well as with any route of administration described herein. Particularly important is the showing herein of intranasal administration of an iRNA agent and its ability to inhibit viral replication in respiratory tissues.

The details of one or more embodiments of the invention are set forth in the accompanying drawings and the description below. Other features, objects, and advantages of the invention will be apparent from this description, the drawings, and from the claims. This application incorporates all cited references, patents, and patent applications by references in their entirety for all purposes.

BRIEF DESCRIPTION OF DRAWINGS

FIG. 1: In vitro inhibition of RSV using iRNA agents. iRNA agents provided in Table 1 (a-c) were tested for anti-RSV activity in a plaque formation assay as described in the Examples. Each column (bar) represents an iRNA agent provided in Table 1 (a-c), e.g. column 1 is the first agent in Table 1a etc. Active iRNA agents were identified.

FIG. 2: In vitro dose response inhibition of RSV using iRNA agents. Examples of active agents from Table 1 were tested for anti-RSV activity in a plaque formation assay as described in the Examples at four concentrations. A dose dependent response was found with active iRNA agent tested.

FIG. 3: In vitro inhibition of RSV B subtype using iRNA agents. iRNA agents provided in FIG. 2 were tested for anti-RSV activity against subtype B in a plaque formation assay as described in the Examples. Subtype B was inhibited by the iRNA agents tested.

FIG. 4: In vivo inhibition of RSV using iRNA agents. Agents as described in the figure were tested for anti-RSV activity in a mouse model as described in the Examples. The iRNA agents were effective at reducing viral titers in vivo.

FIG. 5: In vivo inhibition of RSV using AL-DP-1730. AL-DP-1730 was tested for dose dependent activity using the methods provided in the Examples. The agents showed a dose dependent response.

FIG. 6: In vivo inhibition of RSV using iRNA agents. iRNA agents described in the Figure were tested for anti-RSV activity in vivo as described in the Examples.

FIG. 7: In vivo inhibition of RSV using iRNA agents. iRNA agents described in the Figure were tested for anti-RSV activity in vivo as described in the Examples.

FIG. 8A: In vivo inhibition of RSV using iRNA agents delivered topically.

FIG. 8B: In vivo inhibition of RSV using iRNA agents delivered via aerosol. iRNA agents described in the Figure were tested for anti-RSV activity in vivo as described in the Example.

FIG. 9: In vivo protection against RSV infection using iRNA agents. iRNA agents described in the Figure were tested prior to RSV challenge to test for protective activity.

DETAILED DESCRIPTION

For ease of exposition the term "nucleotide" or "ribonucleotide" is sometimes used herein in reference to one or more monomeric subunits of an RNA agent. It will be understood that the usage of the term "ribonucleotide" or "nucleotide" herein can, in the case of a modified RNA or nucleotide surrogate, also refer to a modified nucleotide, or surrogate replacement moiety, as further described below, at one or more positions.

An "RNA agent" as used herein, is an unmodified RNA, modified RNA, or nucleoside surrogate, all of which are described herein or are well known in the RNA synthetic art. While numerous modified RNAs and nucleoside surrogates are described, preferred examples include

those which have greater resistance to nuclease degradation than do unmodified RNAs. Preferred examples include those that have a 2' sugar modification, a modification in a single strand overhang, preferably a 3' single strand overhang, or, particularly if single stranded, a 5'-modification which includes one or more phosphate groups or one or more analogs of a phosphate group.

An "iRNA agent" (abbreviation for "interfering RNA agent") as used herein, is an RNA agent, which can down-regulate the expression of a target gene, e.g., RSV. While not wishing to be bound by theory, an iRNA agent may act by one or more of a number of mechanisms, including post-transcriptional cleavage of a target mRNA sometimes referred to in the art as RNAi, or pre-transcriptional or pre-translational mechanisms. An iRNA agent can be a double stranded (ds) iRNA agent.

A "ds iRNA agent" (abbreviation for "double stranded iRNA agent"), as used herein, is an iRNA agent which includes more than one, and preferably two, strands in which interchain hybridization can form a region of duplex structure. A "strand" herein refers to a contigouous sequence of nucleotides (including non-naturally occurring or modified nucleotides). The two or more strands may be, or each form a part of, separate molecules, or they may be covalently interconnected, e.g. by a linker, e.g. a polyethyleneglycol linker, to form but one molecule. At least one strand can include a region which is sufficiently complementary to a target RNA. Such strand is termed the "antisense strand". A second strand comprised in the dsRNA agent which comprises a region complementary to the antisense strand is termed the "sense strand". However, a ds iRNA agent can also be formed from a single RNA molecule which is, at least partly; self-complementary, forming, e.g., a hairpin or panhandle structure, including a duplex region. In such case, the term "strand" refers to one of the regions of the RNA molecule that is complementary to another region of the same RNA molecule.

Although, in mammalian cells, long ds iRNA agents can induce the interferon response which is frequently deleterious, short ds iRNA agents do not trigger the interferon response, at least not to an extent that is deleterious to the cell and/or host. The iRNA agents of the present invention include molecules which are sufficiently short that they do not trigger a deleterious

interferon response in mammalian cells. Thus, the administration of a composition of an iRNA agent (*e.g.*, formulated as described herein) to a mammalian cell can be used to silence expression of an RSV gene while circumventing a deleterious interferon response. Molecules that are short enough that they do not trigger a deleterious interferon response are termed siRNA agents or siRNAs herein. "siRNA agent" or "siRNA" as used herein, refers to an iRNA agent, *e.g.*, a ds iRNA agent, that is sufficiently short that it does not induce a deleterious interferon response in a human cell, *e.g.*, it has a duplexed region of less than 30 nucleotide pairs.

The isolated iRNA agents described herein, including ds iRNA agents and siRNA agents, can mediate silencing of a gene, *e.g.*, by RNA degradation. For convenience, such RNA is also referred to herein as the RNA to be silenced. Such a gene is also referred to as a target gene. Preferably, the RNA to be silenced is a gene product of an RSV gene, particularly the P, N or L gene product.

As used herein, the phrase "mediates RNAi" refers to the ability of an agent to silence, in a sequence specific manner, a target gene. "Silencing a target gene" means the process whereby a cell containing and/or secreting a certain product of the target gene when not in contact with the agent, will contain and/or secret at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, or 90% less of such gene product when contacted with the agent, as compared to a similar cell which has not been contacted with the agent. Such product of the target gene can, for example, be a messenger RNA (mRNA), a protein, or a regulatory element.

In the anti viral uses of the present invention, silencing of a target gene will result in a reduction in "viral titer" in the cell or in the subject. As used herein, "reduction in viral titer" refers to a decrease in the number of viable virus produced by a cell or found in an organism undergoing the silencing of a viral target gene. Reduction in the cellular amount of virus produced will preferably lead to a decrease in the amount of measurable virus produced in the tissues of a subject undergoing treatment and a reduction in the severity of the symptoms of the viral infection. iRNA agents of the present invention are also referred to as "antiviral iRNA agents".

As used herein, a "RSV gene" refers to any one of the genes identified in the RSV virus genome (See Falsey, A. R., and E. E. Walsh, 2000, Clinical Microbiological Reviews 13:371-84). These genes are readily known in the art and include the N, P and L genes which are exemplified herein.

As used herein, the term "complementary" is used to indicate a sufficient degree of complementarity such that stable and specific binding occurs between a compound of the invention and a target RNA molecule, e.g. an RSV viral mRNA molecule. Specific binding requires a sufficient degree of complementarity to avoid non-specific binding of the oligomeric compound to non-target sequences under conditions in which specific binding is desired, *i.e.*, under physiological conditions in the case of *in vivo* assays or therapeutic treatment, or in the case of *in vitro* assays, under conditions in which the assays are performed. The non-target sequences typically differ by at least 4 nucleotides.

As used herein, an iRNA agent is "sufficiently complementary" to a target RNA, *e.g.*, a target mRNA (e.g., a target RSV mRNA) if the iRNA agent reduces the production of a protein encoded by the target RNA in a cell. The iRNA agent may also be "exactly complementary" to the target RNA, *e.g.*, the target RNA and the iRNA agent anneal, preferably to form a hybrid made exclusively of Watson-Crick base pairs in the region of exact complementarity. A "sufficiently complementary" iRNA agent can include an internal region (*e.g.*, of at least 10 nucleotides) that is exactly complementary to a target viral RNA. Moreover, in some embodiments, the iRNA agent specifically discriminates a single-nucleotide difference. In this case, the iRNA agent only mediates RNAi if exact complementarity is found in the region (*e.g.*, within 7 nucleotides of) the single-nucleotide difference. Preferred iRNA agents will be based on or consist or comprise the sense and antisense sequences provided in the Examples.

As used herein, "essentially identical" when used referring to a first nucleotide sequence in comparison to a second nucleotide sequence means that the first nucleotide sequence is identical to the second nucleotide sequence except for up to one, two or three nucleotide substitutions (e.g. adenosine replaced by uracil).

As used herein, a "subject" refers to a mammalian organism undergoing treatment for a disorder mediated by viral expression, such as RSV infection or undergoing treatment prophylactically to prevent viral infection. The subject can be any mammal, such as a primate, cow, horse, mouse, rat, dog, pig, goat. In the preferred embodiment, the subject is a human.

As used herein, treating RSV infection refers to the amelioration of any biological or pathological endpoints that 1) is mediated in part by the presence of the virus in the subject and 2) whose outcome can be affected by reducing the level of viral gene products present.

Design and Selection of iRNA agents

The present invention is based on the demonstration of target gene silencing of a respiratory viral gene *in vivo* following local administration to the lungs and nasal passage of an iRNA agent either via intranasal administration/inhalation or systemically/parenterally via injection and the resulting treatment of viral infection. The present invention is further extended to the use of iRNA agents to more than one respiratory virus and the treatment of both virus infections with co-administration of two or more iRNA agents.

Based on these results, the invention specifically provides an iRNA agent that can be used in treating viral infection, particularly respiratory viruses and in particular RSV infection, in isolated form and as a pharmaceutical composition described below. Such agents will include a sense strand having at least 15 or more contiguous nucleotides that are complementary to a viral gene and an antisense strand having at least 15 or more contiguous nucleotides that are complementary to the sense strand sequence. Particularly useful are iRNA agents that consist of, consist essentially of or comprise a nucleotide sequence from the P N and L gene of RSV as provided in Table 1 (a-c).

The iRNA agents of the present invention are based on and comprise at least 15 or more contiguous nucleotides from one of the iRNA agents shown to be active in Table 1 (a-c). In such agents, the agent can consist of consist essentially of or comprise the entire sequence provided in the table or can comprise 15 or more contiguous residues provided in Table1a-c along with additional nucleotides from contiguous regions of the target gene.

An iRNA agent can be rationally designed based on sequence information and desired characteristics and the information provided in Table 1 (a-c). For example, an iRNA agent can be designed according to sequence of the agents provided in the Tables as well as in view of the entire coding sequence of the target gene.

Accordingly, the present invention provides iRNA agents comprising a sense strand and antisense strand each comprising a sequence of at least 15, 16, 17, 18, 19, 20, 21 or 23 nucleotides which is essentially identical to, as defined above, a portion of a gene from a respiratory virus, particularly the P, N or L protein genes of RSV. Exemplified iRNA agents include those that comprise 15 or more contiguous nucleotides from one of the agents provided in Table 1 (a-c).

The antisense strand of an iRNA agent should be equal to or at least, 15, 16 17, 18, 19, 25, 29, 40, or 50 nucleotides in length. It should be equal to or less than 50, 40, or 30, nucleotides in length. Preferred ranges are 15-30, 17 to 25, 19 to 23, and 19 to 21 nucleotides in length. Exemplified iRNA agents include those that comprise 15 or more nucleotides from one of the antisense strands of one of the agents in Table 1 (a-c).

The sense strand of an iRNA agent should be equal to or at least 15, 16 17, 18, 19, 25, 29, 40, or 50 nucleotides in length. It should be equal to or less than 50, 40, or 30 nucleotides in length. Preferred ranges are 15-30, 17 to 25, 19 to 23, and 19 to 21 nucleotides in length. Exemplified iRNA agents include those that comprise 15 or more nucleotides from one of the sense strands of one of the agents in Table 1 (a-c).

The double stranded portion of an iRNA agent should be equal to or at least, 15, 16 17, 18, 19, 20, 21, 22, 23, 24, 25, 29, 40, or 50 nucleotide pairs in length. It should be equal to or less than 50, 40, or 30 nucleotides pairs in length. Preferred ranges are 15-30, 17 to 25, 19 to 23, and 19 to 21 nucleotides pairs in length.

The agents provided in Table 1 (a-c) are 21 nucleotide in length for each strand. The iRNA agents contain a 19 nucleotide double stranded region with a 2 nucleotide overhang on each of the 3' ends of the agent. These agents can be modified as described herein to obtain

equivalent agents comprising at least a portion of these sequences (15 or more contiguous nucleotides) and or modifications to the oligonucleotide bases and linkages.

Generally, the iRNA agents of the instant invention include a region of sufficient complementarity to the viral gene, e.g. the P, N or L protein of RSV, and are of sufficient length in terms of nucleotides, that the iRNA agent, or a fragment thereof, can mediate down regulation of the specific viral gene. The antisense strands of the iRNA agents of the present invention are preferably fully complementary to the mRNA sequences of viral gene, as is herein for the P, L or N proteins of RSV. However, it is not necessary that there be perfect complementarity between the iRNA agent and the target, but the correspondence must be sufficient to enable the iRNA agent, or a cleavage product thereof, to direct sequence specific silencing, *e.g.*, by RNAi cleavage of an RSV mRNA.

Therefore, the iRNA agents of the instant invention include agents comprising a sense strand and antisense strand each comprising a sequence of at least 16, 17 or 18 nucleotides which is essentially identical, as defined below, to one of the sequences of a viral gene, particularly the P, N or L protein of RSV, such as those agent provided in Table 1 (a-c), except that not more than 1, 2 or 3 nucleotides per strand, respectively, have been substituted by other nucleotides (e.g. adenosine replaced by uracil), while essentially retaining the ability to inhibit RSV expression in cultured human cells, as defined below. These agents will therefore possess at least 15 or more nucleotides identical to one of the sequences of a viral gene, particularly the P, L or N protein gene of RSV, but 1, 2 or 3 base mismatches with respect to either the target viral mRNA sequence or between the sense and antisense strand are introduced. Mismatches to the target viral mRNA sequence, particularly in the antisense strand, are most tolerated in the terminal regions and if present are preferably in a terminal region or regions, *e.g.*, within 6, 5, 4, or 3 nucleotides of a 5' and/or 3' terminus, most preferably within 6, 5, 4, or 3 nucleotides of the 5'-terminus of the sense strand or the 3'-terminus of the antisense strand. The sense strand need only be sufficiently complementary with the antisense strand to maintain the overall double stranded character of the molecule.

It is preferred that the sense and antisense strands be chosen such that the iRNA agent includes a single strand or unpaired region at one or both ends of the molecule, such as those exemplified in Table 1 (a-c). Thus, an iRNA agent contains sense and antisense strands, preferably paired to contain an overhang, *e.g.*, one or two 5' or 3' overhangs but preferably a 3' overhang of 2-3 nucleotides. Most embodiments will have a 3' overhang. Preferred siRNA agents will have single-stranded overhangs, preferably 3' overhangs, of 1 to 4, or preferably 2 or 3 nucleotides, in length, on one or both ends of the iRNA agent. The overhangs can be the result of one strand being longer than the other, or the result of two strands of the same length being staggered. 5'-ends are preferably phosphorylated.

Preferred lengths for the duplexed region is between 15 and 30, most preferably 18, 19, 20, 21, 22, and 23 nucleotides in length, *e.g.*, in the siRNA agent range discussed above. Embodiments in which the two strands of the siRNA agent are linked, *e.g.*, covalently linked are also included. Hairpin, or other single strand structures which provide the required double stranded region, and preferably a 3' overhang are also within the invention.

Evaluation of Candidate iRNA Agents

A candidate iRNA agent can be evaluated for its ability to down regulate target gene expression. For example, a candidate iRNA agent can be provided, and contacted with a cell, e.g. a human cell, that has been infected with or will be infected with the virus of interest, e.g., a virus containing the target gene,. Alternatively, the cell can be transfected with a construct from which a target viral gene is expressed, thus preventing the need for a viral infectivity model. The level of target gene expression prior to and following contact with the candidate iRNA agent can be compared, e.g. on an RNA, protein level or viral titer. If it is determined that the amount of RNA, protein or virus expressed from the target gene is lower following contact with the iRNA agent, then it can be concluded that the iRNA agent down-regulates target gene expression. The level of target viral RNA or viral protein in the cell or viral titer in a cell or tissue can be determined by any method desired. For example, the level of target RNA can be determined by Northern blot analysis, reverse transcription coupled with polymerase chain reaction (RT-PCR), bDNA analysis, or RNAse protection assay. The level of protein can be determined, for

example, by Western blot analysis or immuno-fluorescence. Viral titer can be detected through a plaque formation assay.

Stability testing, modification, and retesting of iRNA agents

A candidate iRNA agent can be evaluated with respect to stability, e.g., its susceptibility to cleavage by an endonuclease or exonuclease, such as when the iRNA agent is introduced into the body of a subject. Methods can be employed to identify sites that are susceptible to modification, particularly cleavage, e.g., cleavage by a component found in the body of a subject.

When sites susceptible to cleavage are identified, a further iRNA agent can be designed and/or synthesized wherein the potential cleavage site is made resistant to cleavage, e.g. by introduction of a 2'-modification on the site of cleavage, e.g. a 2'-O-methyl group. This further iRNA agent can be retested for stability, and this process may be iterated until an iRNA agent is found exhibiting the desired stability.

***In Vivo* Testing**

An iRNA agent identified as being capable of inhibiting viral gene expression can be tested for functionality in vivo in an animal model (e.g., in a mammal, such as in mouse, rat or primate) as shown in the examples. For example, the iRNA agent can be administered to an animal, and the iRNA agent evaluated with respect to its biodistribution, stability, and its ability to inhibit viral, e.g. RSV, gene expression or reduce viral titer.

The iRNA agent can be administered directly to the target tissue, such as by injection, or the iRNA agent can be administered to the animal model in the same manner that it would be administered to a human. As shown herein, the agent can be preferably administered via inhalation as a means of treating viral infection.

The iRNA agent can also be evaluated for its intracellular distribution. The evaluation can include determining whether the iRNA agent was taken up into the cell. The evaluation can also include determining the stability (e.g., the half-life) of the iRNA agent. Evaluation of an iRNA agent *in vivo* can be facilitated by use of an iRNA agent conjugated to a traceable marker

(e.g., a fluorescent marker such as fluorescein; a radioactive label, such as ^{35}S, ^{32}P, ^{33}P, or ^{3}H; gold particles; or antigen particles for immunohistochemistry) or other suitable detection method.

The iRNA agent can be evaluated with respect to its ability to down regulate viral gene expression. Levels of viral gene expression *in vivo* can be measured, for example, by *in situ* hybridization, or by the isolation of RNA from tissue prior to and following exposure to the iRNA agent. Where the animal needs to be sacrificed in order to harvest the tissue, an untreated control animal will serve for comparison. Target viral mRNA can be detected by any desired method, including but not limited to RT-PCR, Northern blot, branched-DNA assay, or RNAase protection assay. Alternatively, or additionally, viral gene expression can be monitored by performing Western blot analysis on tissue extracts treated with the iRNA agent or by ELISA. Viral titer can be determined using a pfu assy.

iRNA Chemistry

Described herein are isolated iRNA agents, *e.g.*, ds RNA agents, that mediate RNAi to inhibit expression of a viral gene, e.g. the P protein of RSV.

RNA agents discussed herein include otherwise unmodified RNA as well as RNA which have been modified, *e.g.*, to improve efficacy, and polymers of nucleoside surrogates. Unmodified RNA refers to a molecule in which the components of the nucleic acid, namely sugars, bases, and phosphate moieties, are the same or essentially the same as that which occur in nature, preferably as occur naturally in the human body. The art has referred to rare or unusual, but naturally occurring, RNAs as modified RNAs, see, *e.g.*, Limbach *et al.*, (1994) *Nucleic Acids Res.* 22: 2183-2196. Such rare or unusual RNAs, often termed modified RNAs (apparently because these are typically the result of a post-transcriptional modification) are within the term unmodified RNA, as used herein. Modified RNA as used herein refers to a molecule in which one or more of the components of the nucleic acid, namely sugars, bases, and phosphate moieties, are different from that which occurs in nature, preferably different from that which occurs in the human body. While they are referred to as modified "RNAs," they will of course, because of the modification, include molecules which are not RNAs. Nucleoside surrogates are molecules in which the ribophosphate backbone is replaced with a non-ribophosphate construct

that allows the bases to the presented in the correct spatial relationship such that hybridization is substantially similar to what is seen with a ribophosphate backbone, *e.g.*, non-charged mimics of the ribophosphate backbone. Examples of each of the above are discussed herein.

Modifications described herein can be incorporated into any double-stranded RNA and RNA-like molecule described herein, e.g., an iRNA agent. It may be desirable to modify one or both of the antisense and sense strands of an iRNA agent. As nucleic acids are polymers of subunits or monomers, many of the modifications described below occur at a position which is repeated within a nucleic acid, *e.g.*, a modification of a base, or a phosphate moiety, or the non-linking O of a phosphate moiety. In some cases the modification will occur at all of the subject positions in the nucleic acid but in many, and in fact in most, cases it will not. By way of example, a modification may only occur at a 3' or 5' terminal position, may only occur in a terminal region, *e.g.* at a position on a terminal nucleotide or in the last 2, 3, 4, 5, or 10 nucleotides of a strand. A modification may occur in a double strand region, a single strand region, or in both. *E.g.*, a phosphorothioate modification at a non-linking O position may only occur at one or both termini, may only occur in a terminal regions, *e.g.*, at a position on a terminal nucleotide or in the last 2, 3, 4, 5, or 10 nucleotides of a strand, or may occur in double strand and single strand regions, particularly at termini. Similarly, a modification may occur on the sense strand, antisense strand, or both. In some cases, the sense and antisense strand will have the same modifications or the same class of modifications, but in other cases the sense and antisense strand will have different modifications, *e.g.*, in some cases it may be desirable to modify only one strand, e.g. the sense strand.

Two prime objectives for the introduction of modifications into iRNA agents is their stabilization towards degradation in biological environments and the improvement of pharmacological properties, e.g. pharmacodynamic properties, which are further discussed below. Other suitable modifications to a sugar, base, or backbone of an iRNA agent are described in co-owned PCT Application No. PCT/US2004/01193, filed January 16, 2004. An iRNA agent can include a non-naturally occurring base, such as the bases described in co-owned PCT Application No. PCT/US2004/011822, filed April 16, 2004. An iRNA agent can include a non-naturally occurring sugar, such as a non-carbohydrate cyclic carrier molecule. Exemplary

features of non-naturally occurring sugars for use in iRNA agents are described in co-owned PCT Application No. PCT/US2004/11829 filed April 16, 2003.

An iRNA agent can include an internucleotide linkage (*e.g.*, the chiral phosphorothioate linkage) useful for increasing nuclease resistance. In addition, or in the alternative, an iRNA agent can include a ribose mimic for increased nuclease resistance. Exemplary internucleotide linkages and ribose mimics for increased nuclease resistance are described in co-owned PCT Application No. PCT/US2004/07070 filed on March 8, 2004.

An iRNA agent can include ligand-conjugated monomer subunits and monomers for oligonucleotide synthesis. Exemplary monomers are described in co-owned U.S. Application No. 10/916,185, filed on August 10, 2004.

An iRNA agent can have a ZXY structure, such as is described in co-owned PCT Application No. PCT/US2004/07070 filed on March 8, 2004.

An iRNA agent can be complexed with an amphipathic moiety. Exemplary amphipathic moieties for use with iRNA agents are described in co-owned PCT Application No. PCT/US2004/07070 filed on March 8, 2004.

In another embodiment, the iRNA agent can be complexed to a delivery agent that features a modular complex. The complex can include a carrier agent linked to one or more of (preferably two or more, more preferably all three of): (a) a condensing agent (*e.g.*, an agent capable of attracting, *e.g.*, binding, a nucleic acid, *e.g.*, through ionic or electrostatic interactions); (b) a fusogenic agent (*e.g.*, an agent capable of fusing and/or being transported through a cell membrane); and (c) a targeting group, *e.g.*, a cell or tissue targeting agent, *e.g.*, a lectin, glycoprotein, lipid or protein, *e.g.*, an antibody, that binds to a specified cell type. iRNA agents complexed to a delivery agent are described in co-owned PCT Application No. PCT/US2004/07070 filed on March 8, 2004.

An iRNA agent can have non-canonical pairings, such as between the sense and antisense sequences of the iRNA duplex. Exemplary features of non-canonical iRNA agents are described in co-owned PCT Application No. PCT/US2004/07070 filed on March 8, 2004.

Enhanced nuclease resistance

An iRNA agent, e.g., an iRNA agent that targets RSV, can have enhanced resistance to nucleases.

For increased nuclease resistance and/or binding affinity to the target, an iRNA agent, e.g., the sense and/or antisense strands of the iRNA agent, can include, for example, 2'-modified ribose units and/or phosphorothioate linkages. *E.g.*, the 2′ hydroxyl group (OH) can be modified or replaced with a number of different "oxy" or "deoxy" substituents.

Examples of "oxy"-2′ hydroxyl group modifications include alkoxy or aryloxy (OR, *e.g.*, R = H, alkyl, cycloalkyl, aryl, aralkyl, heteroaryl or sugar); polyethyleneglycols (PEG), $O(CH_2CH_2O)_nCH_2CH_2OR$; "locked" nucleic acids (LNA) in which the 2′ hydroxyl is connected, *e.g.*, by a methylene bridge, to the 4′ carbon of the same ribose sugar; O-AMINE and aminoalkoxy, $O(CH_2)_n$AMINE, (*e.g.*, AMINE = NH_2; alkylamino, dialkylamino, heterocyclyl amino, arylamino, diaryl amino, heteroaryl amino, or diheteroaryl amino, ethylene diamine, polyamino). It is noteworthy that oligonucleotides containing only the methoxyethyl group (MOE), ($OCH_2CH_2OCH_3$, a PEG derivative), exhibit nuclease stabilities comparable to those modified with the robust phosphorothioate modification.

"Deoxy" modifications include hydrogen (*i.e.* deoxyribose sugars, which are of particular relevance to the overhang portions of partially ds RNA); halo (*e.g.*, fluoro); amino (*e.g.* NH_2; alkylamino, dialkylamino, heterocyclyl, arylamino, diaryl amino, heteroaryl amino, diheteroaryl amino, or amino acid); $NH(CH_2CH_2NH)_nCH_2CH_2$-AMINE (AMINE = NH_2; alkylamino, dialkylamino, heterocyclyl amino, arylamino, diaryl amino, heteroaryl amino,or diheteroaryl amino), -NHC(O)R (R = alkyl, cycloalkyl, aryl, aralkyl, heteroaryl or sugar), cyano; mercapto; alkyl-thio-alkyl; thioalkoxy; and alkyl, cycloalkyl, aryl, alkenyl and alkynyl, which may be optionally substituted with *e.g.*, an amino functionality.

Preferred substitutents are 2′-methoxyethyl, 2′-OCH3, 2′-O-allyl, 2′-C- allyl, and 2′-fluoro.

One way to increase resistance is to identify cleavage sites and modify such sites to inhibit cleavage, as described in co-owned U.S. Application No. 60/559,917, filed on May 4, 2004. For example, the dinucleotides 5'-UA-3', 5'-UG-3', 5'-CA-3', 5'-UU-3', or 5'-CC-3' can serve as cleavage sites. Enhanced nuclease resistance can therefore be achieved by modifying the 5' nucleotide, resulting, for example, in at least one 5'-uridine-adenine-3' (5'-UA-3') dinucleotide wherein the uridine is a 2'-modified nucleotide; at least one 5'-uridine-guanine-3' (5'-UG-3') dinucleotide, wherein the 5'-uridine is a 2'-modified nucleotide; at least one 5'-cytidine-adenine-3' (5'-CA-3') dinucleotide, wherein the 5'-cytidine is a 2'-modified nucleotide; at least one 5'-uridine-uridine-3' (5'-UU-3') dinucleotide, wherein the 5'-uridine is a 2'-modified nucleotide; or at least one 5'-cytidine-cytidine-3' (5'-CC-3') dinucleotide, wherein the 5'-cytidine is a 2'-modified nucleotide. The iRNA agent can include at least 2, at least 3, at least 4 or at least 5 of such dinucleotides. In certain embodiments, all the pyrimidines of an iRNA agent carry a 2'-modification, and the iRNA agent therefore has enhanced resistance to endonucleases.

To maximize nuclease resistance, the 2' modifications can be used in combination with one or more phosphate linker modifications (*e.g.*, phosphorothioate). The so-called "chimeric" oligonucleotides are those that contain two or more different modifications.

The inclusion of furanose sugars in the oligonucleotide backbone can also decrease endonucleolytic cleavage. An iRNA agent can be further modified by including a 3' cationic group, or by inverting the nucleoside at the 3'-terminus with a 3'-3' linkage. In another alternative, the 3'-terminus can be blocked with an aminoalkyl group, e.g., a 3' C5-aminoalkyl dT. Other 3' conjugates can inhibit 3'-5' exonucleolytic cleavage. While not being bound by theory, a 3' conjugate, such as naproxen or ibuprofen, may inhibit exonucleolytic cleavage by sterically blocking the exonuclease from binding to the 3'-end of oligonucleotide. Even small alkyl chains, aryl groups, or heterocyclic conjugates or modified sugars (D-ribose, deoxyribose, glucose etc.) can block 3'-5'-exonucleases.

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alkyl chains, aryl groups, or heterocyclic conjugates or modified sugars (D-ribose, deoxyribose, glucose etc.) can block 3'-5'-exonucleases.

An iRNA agent can have increased resistance to nucleases when a duplexed iRNA agent includes a single-stranded nucleotide overhang on at least one end. In preferred embodiments, the nucleotide overhang includes 1 to 4, preferably 2 to 3, unpaired nucleotides. In a preferred embodiment, the unpaired nucleotide of the single-stranded overhang that is directly adjacent to the terminal nucleotide pair contains a purine base, and the terminal nucleotide pair is a G-C pair, or at least two of the last four complementary nucleotide pairs are G-C pairs. In further embodiments, the nucleotide overhang may have 1 or 2 unpaired nucleotides, and in an exemplary embodiment the nucleotide overhang is 5'-GC-3'. In preferred embodiments, the nucleotide overhang is on the 3'-end of the antisense strand. In one embodiment, the iRNA agent includes the motif 5'-CGC-3' on the 3'-end of the antisense strand, such that a 2-nt overhang 5'-GC-3' is formed.

Thus, an iRNA agent can include modifications so as to inhibit degradation, e.g., by nucleases, e.g., endonucleases or exonucleases, found in the body of a subject. These monomers are referred to herein as NRMs, or Nuclease Resistance promoting Monomers, the corresponding modifications as NRM modifications. In many cases these modifications will modulate other properties of the iRNA agent as well, e.g., the ability to interact with a protein, e.g., a transport protein, e.g., serum albumin, or a member of the RISC, or the ability of the first and second sequences to form a duplex with one another or to form a duplex with another sequence, e.g., a target molecule.

One or more different NRM modifications can be introduced into an iRNA agent or into a sequence of an iRNA agent. An NRM modification can be used more than once in a sequence or in an iRNA agent.

NRM modifications include some which can be placed only at the terminus and others which can go at any position. Some NRM modifications that can inhibit hybridization are preferably used only in terminal regions, and more preferably not at the cleavage site or in the cleavage region of a sequence which targets a subject sequence or gene, particularly on the

antisense strand. They can be used anywhere in a sense strand, provided that sufficient hybridization between the two strands of the ds iRNA agent is maintained. In some embodiments it is desirable to put the NRM at the cleavage site or in the cleavage region of a sense strand, as it can minimize off-target silencing.

In most cases, the NRM modifications will be distributed differently depending on whether they are comprised on a sense or antisense strand. If on an antisense strand, modifications which interfere with or inhibit endonuclease cleavage should not be inserted in the region which is subject to RISC mediated cleavage, e.g., the cleavage site or the cleavage region (As described in Elbashir *et al.*, 2001, Genes and Dev. 15: 188, hereby incorporated by reference). Cleavage of the target occurs about in the middle of a 20 or 21 nt antisense strand, or about 10 or 11 nucleotides upstream of the first nucleotide on the target mRNA which is complementary to the antisense strand. As used herein cleavage site refers to the nucleotides on either side of the site of cleavage, on the target mRNA or on the iRNA agent strand which hybridizes to it. Cleavage region means the nucleotides within 1, 2, or 3 nucleotides of the cleavage site, in either direction.

Such modifications can be introduced into the terminal regions, e.g., at the terminal position or with 2, 3, 4, or 5 positions of the terminus, of a sequence which targets or a sequence which does not target a sequence in the subject.

<u>Tethered Ligands</u>

The properties of an iRNA agent, including its pharmacological properties, can be influenced and tailored, for example, by the introduction of ligands, e.g. tethered ligands.

A wide variety of entities, e.g., ligands, can be tethered to an iRNA agent, e.g., to the carrier of a ligand-conjugated monomer subunit. Examples are described below in the context of a ligand-conjugated monomer subunit but that is only preferred, entities can be coupled at other points to an iRNA agent.

Preferred moieties are ligands, which are coupled, preferably covalently, either directly or indirectly via an intervening tether, to the carrier. In preferred embodiments, the ligand is

attached to the carrier *via* an intervening tether. The ligand or tethered ligand may be present on the ligand-conjugated monomer when the ligand-conjugated monomer is incorporated into the growing strand. In some embodiments, the ligand may be incorporated into a "precursor" ligand-conjugated monomer subunit after a "precursor" ligand-conjugated monomer subunit has been incorporated into the growing strand. For example, a monomer having, e.g., an amino-terminated tether, e.g., TAP-$(CH_2)_nNH_2$ may be incorporated into a growing sense or antisense strand. In a subsequent operation, i.e., after incorporation of the precursor monomer subunit into the strand, a ligand having an electrophilic group, e.g., a pentafluorophenyl ester or aldehyde group, can subsequently be attached to the precursor ligand-conjugated monomer by coupling the electrophilic group of the ligand with the terminal nucleophilic group of the precursor ligand-conjugated monomer subunit tether.

In preferred embodiments, a ligand alters the distribution, targeting or lifetime of an iRNA agent into which it is incorporated. In preferred embodiments a ligand provides an enhanced affinity for a selected target, e.g., molecule, cell or cell type, compartment, e.g., a cellular or organ compartment, tissue, organ or region of the body, as, e.g., compared to a species absent such a ligand.

Preferred ligands can improve transport, hybridization, and specificity properties and may also improve nuclease resistance of the resultant natural or modified oligoribonucleotide, or a polymeric molecule comprising any combination of monomers described herein and/or natural or modified ribonucleotides.

Ligands in general can include therapeutic modifiers, e.g., for enhancing uptake; diagnostic compounds or reporter groups e.g., for monitoring distribution; cross-linking agents; nuclease-resistance conferring moieties; and natural or unusual nucleobases. General examples include lipophilic moleculeses, lipids, lectins, steroids (e.g.,uvaol, hecigenin, diosgenin), terpenes (e.g., triterpenes, e.g., sarsasapogenin, Friedelin, epifriedelanol derivatized lithocholic acid), vitamins, carbohydrates(*e.g.*, a dextran, pullulan, chitin, chitosan, inulin, cyclodextrin or hyaluronic acid), proteins, protein binding agents, integrin targeting molecules,polycationics, peptides, polyamines, and peptide mimics.

The ligand may be a naturally occurring or recombinant or synthetic molecule, such as a synthetic polymer, e.g., a synthetic polyamino acid. Examples of polyamino acids include polyamino acid is a polylysine (PLL), poly L-aspartic acid, poly L-glutamic acid, styrene-maleic acid anhydride copolymer, poly(L-lactide-co-glycolied) copolymer, divinyl ether-maleic anhydride copolymer, N-(2-hydroxypropyl)methacrylamide copolymer (HMPA), polyethylene glycol (PEG), polyvinyl alcohol (PVA), polyurethane, poly(2-ethylacrylic acid), N-isopropylacrylamide polymers, or polyphosphazine. Example of polyamines include: polyethylenimine, polylysine (PLL), spermine, spermidine, polyamine, pseudopeptide-polyamine, peptidomimetic polyamine, dendrimer polyamine, arginine, amidine, protamine, cationic moieties, e.g., cationic lipid, cationic porphyrin, quaternary salt of a polyamine, or an alpha helical peptide.

Ligands can also include targeting groups, e.g., a cell or tissue targeting agent, e.g., , a thyrotropin, melanotropin, surfactant protein A, Mucin carbohydrate, a glycosylated polyaminoacid, transferrin, bisphosphonate, polyglutamate, polyaspartate, or an RGD peptide or RGD peptide mimetic.

Ligands can be proteins, e.g., glycoproteins, lipoproteins, e.g. low density lipoprotein (LDL), or albumins, e.g. human serum albumin (HSA), or peptides, e.g., molecules having a specific affinity for a co-ligand, or antibodies *e.g.*, an antibody, that binds to a specified cell type such as a cancer cell, endothelial cell, or bone cell. Ligands may also include hormones and hormone receptors. They can also include non-peptidic species, such as cofactors, multivalent lactose, multivalent galactose, N-acetyl-galactosamine, N-acetyl-glucosamine, multivalent mannose, or multivalent fucose. The ligand can be, for example, a lipopolysaccharide, an activator of p38 MAP kinase, or an activator of NF-κB.

The ligand can be a substance, e.g, a drug, which can increase the uptake of the iRNA agent into the cell, for example, by disrupting the cell's cytoskeleton, e.g., by disrupting the cell's microtubules, microfilaments, and/or intermediate filaments. The drug can be, for example, taxon, vincristine, vinblastine, cytochalasin, nocodazole, japlakinolide, latrunculin A, phalloidin, swinholide A, indanocine, or myoservin.

In one aspect, the ligand is a lipid or lipid-based molecule. Such a lipid or lipid-based molecule preferably binds a serum protein, e.g., human serum albumin (HSA). Other molecules that can bind HSA can also be used as ligands. For example, neproxin or aspirin can be used. A lipid or lipid-based ligand can (a) increase resistance to degradation of the conjugate, (b) increase targeting or transport into a target cell or cell membrane, and/or (c) can be used to adjust binding to a serum protein, e.g., HSA.

A lipid based ligand can be used to modulate, e.g., control the binding of the conjugate to a target tissue. For example, a lipid or lipid-based ligand that binds to HSA more strongly will be less likely to be targeted to the kidney and therefore less likely to be cleared from the body. A lipid or lipid-based ligand that binds to HSA less strongly can be used to target the conjugate to the kidney.

In a preferred embodiment, the lipid based ligand binds HSA. Preferably, it binds HSA with a sufficient affinity such that the conjugate will be preferably distributed to a non-kidney tissue. However, it is preferred that the affinity not be so strong that the HSA-ligand binding cannot be reversed.

In another aspect, the ligand is a moiety, e.g., a vitamin or nutrient, which is taken up by a target cell, e.g., a proliferating cell. These are particularly useful for treating disorders characterized by unwanted cell proliferation, e.g., of the malignant or non-malignant type, e.g., cancer cells. Exemplary vitamins include vitamin A, E, and K. Other exemplary vitamins include the B vitamins, e.g., folic acid, B12, riboflavin, biotin, pyridoxal or other vitamins or nutrients taken up by cancer cells.

In another aspect, the ligand is a cell-permeation agent, preferably a helical cell-permeation agent. Preferably, the agent is amphipathic. An exemplary agent is a peptide such as tat or antennapedia. If the agent is a peptide, it can be modified, including a peptidylmimetic, invertomers, non-peptide or pseudo-peptide linkages, and use of D-amino acids. The helical agent is preferably an alpha-helical agent, which preferably has a lipophilic and a lipophobic phase.

5'-Phosphate modifications

In preferred embodiments, iRNA agents are 5' phosphorylated or include a phosphoryl analog at the 5' prime terminus. 5'-phosphate modifications of the antisense strand include those which are compatible with RISC mediated gene silencing. Suitable modifications include: 5'-monophosphate ((HO)2(O)P-O-5'); 5'-diphosphate ((HO)2(O)P-O-P(HO)(O)-O-5'); 5'-triphosphate ((HO)2(O)P-O-(HO)(O)P-O-P(HO)(O)-O-5'); 5'-guanosine cap (7-methylated or non-methylated) (7m-G-O-5'-(HO)(O)P-O-(HO)(O)P-O-P(HO)(O)-O-5'); 5'-adenosine cap (Appp), and any modified or unmodified nucleotide cap structure. Other suitable 5'-phosphate modifications will be knoen to the skilled person.

The sense strand can be modified in order to inactivate the sense strand and prevent formation of an active RISC, thereby potentially reducing off-target effects. This can be accomplished by a modification which prevents 5'-phosphorylation of the sense strand, *e.g.*, by modification with a 5'-O-methyl ribonucleotide (see Nykänen *et al.*, (2001) ATP requirements and small interfering RNA structure in the RNA interference pathway. Cell 107, 309-321.) Other modifications which prevent phosphorylation can also be used, *e.g.*, simply substituting the 5'-OH by H rather than O-Me. Alternatively, a large bulky group may be added to the 5'-phosphate turning it into a phosphodiester linkage.

Delivery of iRNA agents to tissues and cells

Formulation

The iRNA agents described herein can be formulated for administration to a subject, preferably for administration locally to the lungs and nasal passage (respiratory tissues) via inhalation or intranasally administration, or parenterally, e.g. via injection.

For ease of exposition, the formulations, compositions, and methods in this section are discussed largely with regard to unmodified iRNA agents. It should be understood, however, that these formulations, compositions, and methods can be practiced with other iRNA agents, *e.g.*, modified iRNA agents, and such practice is within the invention.

A formulated iRNA agent composition can assume a variety of states. In some examples, the composition is at least partially crystalline, uniformly crystalline, and/or anhydrous (*e.g.*, less than 80, 50, 30, 20, or 10% water). In another example, the iRNA agent is in an aqueous phase, *e.g.*, in a solution that includes water, this form being the preferred form for administration via inhalation.

The aqueous phase or the crystalline compositions can be incorporated into a delivery vehicle, *e.g.*, a liposome (particularly for the aqueous phase), or a particle (*e.g.*, a microparticle as can be appropriate for a crystalline composition). Generally, the iRNA agent composition is formulated in a manner that is compatible with the intended method of administration.

An iRNA agent preparation can be formulated in combination with another agent, *e.g.*, another therapeutic agent or an agent that stabilizes an iRNA agent, *e.g.*, a protein that complexes with the iRNA agent to form an iRNP. Still other agents include chelators, *e.g.*, EDTA (*e.g.*, to remove divalent cations such as Mg^{2+}), salts, RNAse inhibitors (*e.g.*, a broad specificity RNAse inhibitor such as RNAsin) and so forth.

In one embodiment, the iRNA agent preparation includes another iRNA agent, *e.g.*, a second iRNA agent that can mediate RNAi with respect to a second gene. Still other preparations can include at least three, five, ten, twenty, fifty, or a hundred or more different iRNA species. In some embodiments, the agents are directed to the same virus but different target sequences. In another embodiment, each iRNA agents is directed to a different virus. As demonstrated in the Example, more than one virus can be inhibited by co-administering two iRNA agents simultaneously, or at closely time intervals, each one directed to one of the viruses being treated.

Treatment Methods and Routes of Delivery

A composition that includes an iRNA agent of the present invention, e.g., an iRNA agent that targets RSV, can be delivered to a subject by a variety of routes. Exemplary routes include inhalation, intravenous, nasal, or oral delivery. The preferred means of administering the iRNA

agents of the present invention is through direct administration to the lungs and nasal passage or systemically through parental administration.

An iRNA agent can be incorporated into pharmaceutical compositions suitable for administration. For example, compositions can include one or more iRNA agents and a pharmaceutically acceptable carrier. As used herein the language "pharmaceutically acceptable carrier" is intended to include any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like, compatible with pharmaceutical administration. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active compound, use thereof in the compositions is contemplated. Supplementary active compounds can also be incorporated into the compositions.

The pharmaceutical compositions of the present invention may be administered in a number of ways depending upon whether local or systemic treatment is desired and upon the area to be treated. Administration may be topical (including intranasal or intrapulmonary), oral or parenteral. Parenteral administration includes intravenous drip, subcutaneous, intraperitoneal or intramuscular injection.

In general, the delivery of the iRNA agents of the present invention is done to achieve delivery into the subject to the site of infection. The preferred means of achieving this is through either a local administration to the lungs or nasal passage, e.g. into the respiratory tissues via inhalation, nebulization or intranasal administration, or via systemic administration, e.g. parental administration.

Formulations for inhalation or parenteral administration are well known in the art. Such formulation may include sterile aqueous solutions which may also contain buffers, diluents and other suitable additives, an example being PBS or Dextrose 5% in water. For intravenous use, the total concentration of solutes should be controlled to render the preparation isotonic.

The active compounds disclosed herein are preferably administered to the lung(s) or nasal passage of a subject by any suitable means. Active compounds may be administered by

administering an aerosol suspension of respirable particles comprised of the active compound or active compounds, which the subject inhales. The active compound can be aerosolized in a variety of forms, such as, but not limited to, dry powder inhalants, metered dose inhalants, or liquid/liquid suspensions. The respirable particles may be liquid or solid. The particles may optionally contain other therapeutic ingredients such as amiloride, benzamil or phenamil, with the selected compound included in an amount effective to inhibit the reabsorption of water from airway mucous secretions, as described in U.S. Pat. No. 4,501,729.

The particulate pharmaceutical composition may optionally be combined with a carrier to aid in dispersion or transport. A suitable carrier such as a sugar (i.e., dextrose, lactose, sucrose, trehalose, mannitol) may be blended with the active compound or compounds in any suitable ratio (e.g., a 1 to 1 ratio by weight).

Particles comprised of the active compound for practicing the present invention should include particles of respirable size, that is, particles of a size sufficiently small to pass through the mouth or nose and larynx upon inhalation and into the bronchi and alveoli of the lungs. In general, particles ranging from about 1 to 10 microns in size (more particularly, less than about 5 microns in size) are respirable. Particles of non-respirable size which are included in the aerosol tend to deposit in the throat and be swallowed, and the quantity of non-respirable particles in the aerosol is preferably minimized. For nasal administration, a particle size in the range of 10-500 micons is preferred to ensure retention in the nasal cavity.

Liquid pharmaceutical compositions of active compound for producing an aerosol may be prepared by combining the active compound with a suitable vehicle, such as sterile pyrogen free water. The hypertonic saline solutions used to carry out the present invention are preferably sterile, pyrogen-free solutions, comprising from one to fifteen percent (by weight) of the physiologically acceptable salt, and more preferably from three to seven percent by weight of the physiologically acceptable salt.

Aerosols of liquid particles comprising the active compound may be produced by any suitable means, such as with a pressure-driven jet nebulizer or an ultrasonic nebulizer. See, e.g., U.S. Pat. No. 4,501,729. Nebulizers are commercially available devices which transform

solutions or suspensions of the active ingredient into a therapeutic aerosol mist either by means of acceleration of compressed gas, typically air or oxygen, through a narrow venturi orifice or by means of ultrasonic agitation.

Suitable formulations for use in nebulizers consist of the active ingredient in a liquid carrier, the active ingredient comprising up to 40% w/w of the formulation, but preferably less than 20% w/w. The carrier is typically water (and most preferably sterile, pyrogen-free water) or a dilute aqueous alcoholic solution, preferably made isotonic, but may be hypertonic with body fluids by the addition of, for example, sodium chloride. Optional additives include preservatives if the formulation is not made sterile, for example, methyl hydroxybenzoate, antioxidants, flavoring agents, volatile oils, buffering agents and surfactants.

Aerosols of solid particles comprising the active compound may likewise be produced with any solid particulate therapeutic aerosol generator. Aerosol generators for administering solid particulate therapeutics to a subject produce particles which are respirable and generate a volume of aerosol containing a predetermined metered dose of a therapeutic at a rate suitable for human administration. One illustrative type of solid particulate aerosol generator is an insufflator. Suitable formulations for administration by insufflation include finely comminuted powders which may be delivered by means of an insufflator or taken into the nasal cavity in the manner of a snuff. In the insufflator, the powder (e.g., a metered dose thereof effective to carry out the treatments described herein) is contained in capsules or cartridges, typically made of gelatin or plastic, which are either pierced or opened in situ and the powder delivered by air drawn through the device upon inhalation or by means of a manually-operated pump. The powder employed in the insufflator consists either solely of the active ingredient or of a powder blend comprising the active ingredient, a suitable powder diluent, such as lactose, and an optional surfactant. The active ingredient typically comprises from 0.1 to 100 w/w of the formulation.

A second type of illustrative aerosol generator comprises a metered dose inhaler. Metered dose inhalers are pressurized aerosol dispensers, typically containing a suspension or solution formulation of the active ingredient in a liquefied propellant. During use these devices discharge

the formulation through a valve adapted to deliver a metered volume, typically from 10 to 200 ul, to produce a fine particle spray containing the active ingredient. Suitable propellants include certain chlorofluorocarbon compounds, for example, dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane and mixtures thereof. The formulation may additionally contain one or more co-solvents, for example, ethanol, surfactants, such as oleic acid or sorbitan trioleate, antioxidant and suitable flavoring agents.

Administration can be provided by the subject or by another person, *e.g.*, a caregiver. A caregiver can be any entity involved with providing care to the human: for example, a hospital, hospice, doctor's office, outpatient clinic; a healthcare worker such as a doctor, nurse, or other practitioner; or a spouse or guardian, such as a parent. The medication can be provided in measured doses or in a dispenser which delivers a metered dose.

The term "therapeutically effective amount" is the amount present in the composition that is needed to provide the desired level of drug in the subject to be treated to give the anticipated physiological response. In one embodiment, therapeutically effective amounts of two or more iRNA agents, each one directed to a different respiratory virus, e.g. RSV, are administered concurrently to a subject.

The term "physiologically effective amount" is that amount delivered to a subject to give the desired palliative or curative effect.

The term "pharmaceutically acceptable carrier" means that the carrier can be taken into the lungs with no significant adverse toxicological effects on the lungs.

The term "co-administration" refers to administering to a subject two or more agents, and in particular two or more iRNA agents. The agents can be contained in a single pharmaceutical composition and be administered at the same time, or the agents can be contained in separate formulation and administered serially to a subject. So long as the two agents can be detected in the subject at the same time, the two agents are said to be co-administered.

The types of pharmaceutical excipients that are useful as carrier include stabilizers such as human serum albumin (HSA), bulking agents such as carbohydrates, amino acids and

polypeptides; pH adjusters or buffers; salts such as sodium chloride; and the like. These carriers may be in a crystalline or amorphous form or may be a mixture of the two.

Bulking agents that are particularly valuable include compatible carbohydrates, polypeptides, amino acids or combinations thereof. Suitable carbohydrates include monosaccharides such as galactose, D-mannose, sorbose, and the like; disaccharides, such as lactose, trehalose, and the like; cyclodextrins, such as 2-hydroxypropyl-.beta.-cyclodextrin; and polysaccharides, such as raffinose, maltodextrins, dextrans, and the like; alditols, such as mannitol, xylitol, and the like. A preferred group of carbohydrates includes lactose, threhalose, raffinose maltodextrins, and mannitol. Suitable polypeptides include aspartame. Amino acids include alanine and glycine, with glycine being preferred.

Suitable pH adjusters or buffers include organic salts prepared from organic acids and bases, such as sodium citrate, sodium ascorbate, and the like; sodium citrate is preferred.

Dosage. An iRNA agent can be administered at a unit dose less than about 75 mg per kg of bodyweight, or less than about 70, 60, 50, 40, 30, 20, 10, 5, 2, 1, 0.5, 0.1, 0.05, 0.01, 0.005, 0.001, or 0.0005 mg per kg of bodyweight, and less than 200 nmol of iRNA agent (e.g., about 4.4 x 1016 copies) per kg of bodyweight, or less than 1500, 750, 300, 150, 75, 15, 7.5, 1.5, 0.75, 0.15, 0.075, 0.015, 0.0075, 0.0015, 0.00075, 0.00015 nmol of iRNA agent per kg of bodyweight. The unit dose, for example, can be administered by an inhaled dose or nebulization or by injection. In one example, dosage ranges of .02-25 mg/kg is used.

Delivery of an iRNA agent directly to the lungs or nasal passage can be at a dosage on the order of about 1 mg to about 150 mg/nasal passage.

The dosage can be an amount effective to treat or prevent a disease or disorder.

In one embodiment, the unit dose is administered once a day. In other usage, a unit dose is administered twice the first day and then daily. Alternatively, unit dosing can be less than once a day, *e.g.*, less than every 2, 4, 8 or 30 days. In another embodiment, the unit dose is not administered with a frequency (*e.g.*, not a regular frequency). For example, the unit dose may be administered a single time. Because iRNA agent mediated silencing can persist for several days

after administering the iRNA agent composition, in many instances, it is possible to administer the composition with a frequency of less than once per day, or, for some instances, only once for the entire therapeutic regimen.

In one embodiment, a subject is administered an initial dose, and one or more maintenance doses of an iRNA agent, *e.g.*, a double-stranded iRNA agent, or siRNA agent, (*e.g.*, a precursor, *e.g.*, a larger iRNA agent which can be processed into an siRNA agent, or a DNA which encodes an iRNA agent, *e.g.*, a double-stranded iRNA agent, or siRNA agent, or precursor thereof). The maintenance dose or doses are generally lower than the initial dose, *e.g.*, one-half less of the initial dose. A maintenance regimen can include treating the subject with a dose or doses ranging from 0.01 µg to 75 mg/kg of body weight per day, *e.g.*, 70, 60, 50, 40, 30, 20, 10, 5, 2, 1, 0.5, 0.1, 0.05, 0.01, 0.005, 0.001, or 0.0005 mg per kg of bodyweight per day. The maintenance doses are preferably administered no more than once every 5-14 days. Further, the treatment regimen may last for a period of time which will vary depending upon the nature of the particular disease, its severity and the overall condition of the patient. In preferred embodiments the dosage may be delivered no more than once per day, *e.g.*, no more than once per 24, 36, 48, or more hours, *e.g.*, no more than once every 5 or 8 days. Following treatment, the patient can be monitored for changes in his condition and for alleviation of the symptoms of the disease state. The dosage of the compound may either be increased in the event the patient does not respond significantly to current dosage levels, or the dose may be decreased if an alleviation of the symptoms of the disease state is observed, if the disease state has been ablated, or if undesired side-effects are observed.

In one embodiment, the iRNA agent pharmaceutical composition includes a plurality of iRNA agent species. The iRNA agent species can have sequences that are non-overlapping and non-adjacent with respect to a naturally occurring target sequence, e.g., a target sequence of the RSV gene. In another embodiment, the plurality of iRNA agent species is specific for different naturally occurring target genes. For example, an iRNA agent that targets the P protein gene of RSV can be present in the same pharmaceutical composition as an iRNA agent that targets a different gene, for example the N protein gene. In another embodiment, the iRNA agents are specific for different viruses, e.g. RSV.

The concentration of the iRNA agent composition is an amount sufficient to be effective in treating or preventing a disorder or to regulate a physiological condition in humans. The concentration or amount of iRNA agent administered will depend on the parameters determined for the agent and the method of administration, *e.g.* nasal, buccal, or pulmonary. For example, nasal formulations tend to require much lower concentrations of some ingredients in order to avoid irritation or burning of the nasal passages. It is sometimes desirable to dilute an oral formulation up to 10-100 times in order to provide a suitable nasal formulation.

Certain factors may influence the dosage required to effectively treat a subject, including but not limited to the severity of the disease or disorder, previous treatments, the general health and/or age of the subject, and other diseases present. It will also be appreciated that the effective dosage of an iRNA agent such as an siRNA agent used for treatment may increase or decrease over the course of a particular treatment. Changes in dosage may result and become apparent from the results of diagnostic assays. For example, the subject can be monitored after administering an iRNA agent composition. Based on information from the monitoring, an additional amount of the iRNA agent composition can be administered.

The invention is further illustrated by the following examples, which should not be construed as further limiting.

EXAMPLES

Designing antiviral siRNAs against RSV mRNA

siRNA against RSV P, N and L mRNA were synthesized chemically using know procedures. The siRNA sequences and some inhibition cross-subtype activity and IC50 values are listed (Table 1 (a-c)).

In vitro assay and Virus infection

Vero E6 cells were cultured to 80% confluency in DMEM containing 10% heat-inactivated FBS. For siRNA introduction, 4 µl of Transit-TKO was added to 50 µl of serum-free DMEM and incubated at room temperature for 10 minutes. Then, indicated concentration of

siRNA was added to media/TKO reagent respectively and incubated at room temperature for 10 minutes. RNA mixture was added to 200 μl of DMEM containing 10% FBS and then to cell monolayer. Cells were incubated at 37°C, 5% CO_2 for 6 hours. RNA mixture was removed by gentle washing with 1x Hank's Balanced Salt Solutions (HBSS) and 300 plaque-forming units (pfu) per well of RSV/A2 (MOI = 30) was added to wells and adsorbed for 1 hour at 37°C, 5% CO_2. Virus was removed and cells were washed with 1x HBSS. Cells were overlaid with 1% methylcellulose in DMEM containing 10% FBS media, and incubated for 6 days at 37°C, 5% CO_2. Cells were immunostained for plaques using anti-F protein monoclonal antibody 131-2A.

siRNA delivery and virus infection *in vivo*

Pathogen-free 4 week old female BALB/c mice were purchased from Harlan. Mice were under anesthesia during infection and intranasal instillation (i.n.). Mice were immunized by intranasal instillation with indicated amount of siRNA, either uncomplexed, or complexed with 5 ul Transit TKO. 150 μg of Synagis (monoclonal antibody clone 143-6C, anti-RSV F protein) and Mouse Isotype control (IgG1) were administered intraperitoneal (i.p.) four hours prior to RSV challenge (10^6 PFU of RSV/A2). Ten mice per group were used. Animal weights were monitored at days 0, 2, 4, and 6 post-infection. Lungs were harvested at day 6 post-infection, and assayed for RSV by immunostaining plaque assay.

Immunostaining Plaque Assay

24-well plates of Vero E6 cells were cultured to 90% confluency in DMEM containing 10% heat inactivated FBS. Mice lungs were homogenized with hand-held homogenizer in 1 ml sterile Dulbecco's PBS (D-PBS) and 10 fold diluted in serum-free DMEM. Virus containing lung lysate dilutions were plated onto 24 well plates in triplicate and adsorbed for 1 hour at 37°C, 5% CO_2. Wells were overlaid with 1% methylcellulose in DMEM containing 10% FBS. Then, plates were incubated for 6 days at 37°C, 5% CO_2. After 6 days, overlaid media was removed and cells were fixed in acetone:methanol (60:40) for 15 minutes. Cells were blocked with 5% dry Milk/PBS for 1 hour at 37°C. 1:500 dilution of anti-RSV F protein antibody (131-2A) was added to wells and incubated for 2 hours at 37°C. Cells were washed twice in PBS/0.5% Tween 20. 1:500 dilution of goat anti-mouse IgG-Alkaline Phosphatase was added to wells and incubated

for 1 hour at 37°C. Cells were washed twice in PBS/0.5% Tween 20. Reaction was developed using Vector's Alkaline Phosphatase substrate kit II (Vector Black), and counterstained with Hematoxylin. Plaques were visualized and counted using an Olympus Inverted microscope.

Treatment assay

Mice were challenged with RSV (10^6 PFU of RSV/A2) by intranasal instillation at day 0 and treated with 50 ug of indicated siRNA, delivered by intranasal instillation, at the indicated times (day 1-4 post viral challenge). 3-5 mice per group were used and viral titers were measured from lung lysates at day 5 post viral challenge, as previously described.

In vitro inhibition of RSV using iRNA agents.

iRNA agents provided in Table 1 (a-c) were tested for anti-RSV activity in a plaque formation assay as described above (Figure 1). Each column (bar) represents an iRNA agent provided in Table 1 (a-c), e.g. column 1 is the first agent in Table 1a, second column is the second agent and so on. Active iRNA agents were identified by the % of virus remaining. Several agents were identified that showed as much as 90% inhibition. The results are summarized in Table 1 (a-c).

In vitro dose response inhibition of RSV using iRNA agents was determined. Examples of active agents from Table 1 were tested for anti-RSV activity in a plaque formation assay as described above at four concentrations. A dose dependent response was found with active iRNA agent tested (Figure 2) and is summarized in Tables 1(a-c).

In vitro inhibition of RSV B subtype using iRNA agents was tested as described above. iRNA agents provided in Figure 2 were tested for anti-RSV activity against subtype B (Figure 3). RSV subtype B was inhibited by the iRNA agents tested to varying degrees and is summarized in Table 1(a-c).

In vivo inhibition of RSV using iRNA agents.

In vivo inhibition of RSV using AL1729 and AL1730 was tested as described above. Agents as described in Figure 4 were tested for anti-RSV activity in a mouse model. The iRNA agents were effective at reducing viral titers in vivo and more effective than a control antibody (Mab 143-6c, a mouse IgG1 Ab that is approved for RSV treatment).

AL1730 was tested for dose dependent activity using the methods provided above. The agents showed a dose dependent response (Figure 5).

iRNA agents showing in vitro activity were tested for anti-RSV activity in vivo as outlined above. Several agents showed a reduction in viral titers of >4 logs when given prophylactically (Figure 6).

iRNA agents showing in vitro and/or in vivo activity were tested for anti-RSV activity in vivo as in the treatment protocol outlined above. Several agents showed a reduction in viral titers of 2-3 logs (Figure 7) when given 1-2 days following viral infection.

Sequence analysis of isolates across target sequence

Method:

Growth of isolates and RNA isolation: Clinical isolates from RSV infected patients were obtained from Larry Anderson at the CDC in Atlanta Georgia (4 strains) and John DeVincenzo at the University of Tenn., Memphis (15 strains). When these were grown in HEp-2, human epithelial cells (ATCC, Cat# CCL-23) cells, it was noted that the 4 isolates from Georgia were slower growing than the 15 strains from Tennessee; hence, these were processed and analyzed separately. The procedure is briefly described as follows:

Vero E6, monkey kidney epithelial cells (ATCC, Cat# CRL-1586) were grown to 95% confluency and infected with a 1/10 dilution of primary isolates. The virus was absorbed for 1 hour at 37 °C, then cells were supplemented with D-MEM and incubated at 37 °C. On a daily basis, cells were monitored for cytopathic effect (CPE) by light microscopy. At 90% CPE, the

cells were harvested by scraping and pelleted by centrifugation at 3000 rpm for 10 minutes. RNA preparations were performed by standard procedures according to manufacturer's protocol.

Amplification of RSV N gene: Viral RNAs were collected post-infection and used as templates in PCR reactions, using primers that hybridize upstream and downstream of the ALDP-2017 target site to amplify an ~450 bp fragment. Total RNA was denatured at

65 °C for 5 minutes in the presence of forward and reverse RSV N gene primers, stored on ice, and then reverse-transcribed with Superscript III (Invitrogen) for 60 minutes at 55 °C and for 15 minutes at 70 °C. PCR products were analyzed by gel electrophoresis on a 1% agarose gel and purified by standard protocols.

Results: Sequence analysis of the first 15 isolates confirmed that the target site for ALDP-2017 was completely conserved across every strain. Importantly, this conservation was maintained across the diverse populations, which included isolates from both RSV A and B subtypes. Interestingly, when the 4 slower-growing isolates were analyzed, we observed that one of the 4 (LAP6824) had a single base mutation in the ALDP-2017 recognition site. This mutation changed the coding sequence at position 13 of the RSV N gene in this isolate from an A to a G.

Conclusions:

From 19 patient isolates, the sequence of the RSV N gene at the target site for ALDP-2017 has been determined. In 18 of 19 cases (95%), the recognition element for ALDP-2017 is 100% conserved. In one of the isolates, there is a single base alteration changing the nucleotide at position 13 from an A to a G within the RSV N gene. This alteration creates a single G:U wobble between the antisense strand of ALDP-2017 and the target sequence. Based on an understanding of the hybridization potential of such a G:U wobble, it is predicted that ALDP-2017 will be effective in silencing the RSV N gene in this isolate.

Silencing data on isolates

Methods

Vero E6 cells were cultured to 80% confluency in DMEM containing 10% heat-inactivated FBS. For siRNA introduction, 4 µl of Transit-TKO was added to 50 µl of serum-free DMEM and incubated at room temperature for 10 minutes. Then, indicated concentration of siRNA was added to media/TKO reagent respectively and incubated at room temperature for 10 minutes. RNA mixture was added to 200 µl of DMEM containing 10% FBS and then to cell monolayer. Cells were incubated at 37°C, 5% CO_2 for 6 hours. RNA mixture was removed by gentle washing with 1x Hank's Balanced Salt Solutions (HBSS) and 300 plaque-forming units (pfu) per well of RSV/A2 (MOI = 30) was added to wells and adsorbed for 1 hour at 37°C, 5% CO_2. Virus was removed and cells were washed with 1x HBSS. Cells were overlaid with 1% methylcellulose in DMEM containing 10% FBS media, and incubated for 6 days at 37°C, 5% CO_2. Cells were immunostained for plaques using anti-F protein monoclonal antibody 131-2A.

Results: Silencing was seen for all isolates (Table 2)

Isolate name	2017 %plaques remaining	2153 %plaques remaining
RSV/A2	4.49	80.34
RSV/96	5.36	87.50
RSV/87	10.20	79.59
RSV/110	5.41	81.08
RSV/37	4.80	89.60
RSV/67	2.22	91.67
RSV/121	6.25	82.50
RSV/31	4.03	96.77

RSV/38	2.00	92.67
RSV/98	5.13	91.03
RSV/124	3.74	90.37
RSV/95	7.32	64.02
RSV/32	5.45	92.73
RSV/91	8.42	95.79
RSV/110	12.07	94.83
RSV/54	1.90	89.87
RSV/53	7.41	94.07
RSV/33	7.69	95.19

Table 2

Conclusion: All clinical isolates tested were specifically inhibited by siRNA 2017 by greater than 85%. No isolates were significantly inhibited the mismatch control siRNA 2153.

<u>Silencing in plasmid based assay</u>

Method

A 24-well plate is seeded with HeLa S6 cells and grown to 80 % confluence. For each well, mix 1 ug of RSV N-V5 plasmid with siRNA (at indicated concentration), in 50 ul OPTI-MEM and add to Lipofectamine 2000 (Invitrogen)-Optimem mixture prepared according to manufacturer's instructions, and let sit 20 minutes at r.t. to form complex. Add complex to cells and incubate 37°C overnight. Remove the media, wash the cells with PBS and lyse with 50 ul Lysis buffer (RIPA buffer (50mM Tris-HCl pH 8.0, 150 mM NaCl, 1mM EDTA, 0.5% Na deoxycholate, 1% NP-40, 0.05% SDS) for 1-2 min. Inhibition is quantified by measuring the level of RSV protein in cell lysates, detected by western blotting with an anti-V5 antibody

Results: Transient plasmid expression was shown to be an effective assay for RNAi agents (Table 3).

			Protein %	Activity%
1	ALDP2017	10nM	0	100
2		1nM	0	100
3		100pM	0	100
4		10pM	11.78	88.22
5		1pM	70.63	29.37
6		100fM	72.7	27.3
7	Control	PBS	100	0
8	2153	10nM	94.54	4.5

Table 3

Conclusions

siRNA 2017 specifically and dose dependently inhibits the production of RSV N protein when transiently cotranfected with plasmid expressing the RSV N gene. Inhibition is not observed with mismatch control siRNA 2153.

Silencing of RSV via aerosol delivery of siRNA

Method

A 2 mg/ml solution of ALDP-1729 or ALDP-1730 is delivered via nebulization using an aerosol device for a total of 60 sec. Virus was prepared from lung as described above and measured by an ELISA instead of a plaque assay. The ELISA measures the concentration of the RSV N protein in cells infected with virus obtained from mouse lung lysates.

ELISA

Lung lystate is diluted 1:1 with carbonate-bicarbonate buffer ($NaHCO_3$ pH 9.6) to a working concentration of 6-10μg/100μL, added to each test well and incubated at 37°C for 1 hour or overnight at 4°C. Wells washed 3 X with PBS/0.5% Tween 20 then blocked with 5% dry milk/PBS for 1 hour at 37°C or overnight at 4°C. Primary antibody (F protein positive control = clone 131-2A; G protein positive control = 130-2G; negative control = normal IgG1,(BD Pharmingen, cat. #553454, test sera, or hybridoma supernatant) is added to wells at 1:1000 and incubated at 37°C for 1 hour or overnight at 4°C. Wells washed 3 X with PBS/0.5% Tween 20. Secondary antibody (Goat Anti-mouse IgG (H+L) whole molecule-alkaline phosphatase conjugated) diluted 1:1000 to wells (100 μl/well) is added and incubated at 37°C for 1 hour or overnight at 4°C. Wash 3 X with PBS/0.5% Tween 20 then add Npp (Sigmafast) substrate Sigma Aldrich N2770 accordingly to manufacturers instructions. Add 200 μl of substrate/well and incubate for 10-15. Measure absorbance at OD 405/495.

Conclusion

Delivery of RSV specific siRNA decreases the levels of RSV N protein in mouse lungs as compared to the mismatch control siRNA (Figure 8a-b).

In vivo inhibition at day -3-prophylaxis

Method

In vivo prophylaxis was tested using the in vivo method described above except that the siRNA is delivered at different times prior to infection with RSV from 3 days before to 4 hrs before.

Results

siRNA delivered intranasally up to 3 days prior to viral challenge show significant silencing *in vivo* (Figure 9).

Table 1 (a-c). siRNA sequences

Table 1a. RSV L gene

Actual start	Whitehead Start Pos	Sense	Antisense	AL-DP #	% inh RSV A2 (5nM)	% inh RSV A2 500 pM	% inh RSV A2 50 pM	% inh RSV A2 5pM	% inh RSV B (5nM)
3	1	GGAUCCCAUUAUUAAUGGAdTdT	UCCAUUAAUAAUGGGAUCCdTdT	AL-DP-2024	92				
4	2	GAUCCCAUUAUUAAUGGAAdTdT	UUCCAUUAAUAAUGGGAUCdTdT	AL-DP-2026	82				
49	47	AGUUAUUUAAAAGGUGUUAdTdT	UAACACCUUUUAAAUAACUdTdT	AL-DP-2116					
50	48	GUUAUUUAAAAGGUGUUAUdTdT	AUAACACCUUUUAAAUAACdTdT	AL-DP-2117					
53	51	AUUUAAAAGGUGUUAUCUCdTdT	GAGAUAACACCUUUUAAAUdTdT	AL-DP-2118					
55	53	UUAAAAGGUGUUAUCUCUUdTdT	AAGAGAUAACACCUUUUAAdTdT	AL-DP-2119					
156	154	AAGUCCACUACUAGAGCAUdTdT	AUGCUCUAGUAGUGGACUUdTdT	AL-DP-2027	86				
157	155	AGUCCACUACUAGAGCAUAdTdT	UAUGCUCUAGUAGUGGACUdTdT	AL-DP-2028	90				
158	156	GUCCACUACUAGAGCAUAUdTdT	AUAUGCUCUAGUAGUGGACdTdT	AL-DP-2029	89				
159	157	UCCACUACUAGAGCAUAUGdTdT	CAUAUGCUCUAGUAGUGGAdTdT	AL-DP-2030	86				
341	339	GAAGAGCUAUAGAAAUAAGdTdT	CUUAUUUCUAUAGCUCUUCdTdT	AL-DP-2120					
344	342	GAGCUAUAGAAAUAAGUGAdTdT	UCACUUAUUUCUAUAGCUCdTdT	AL-DP-2121					
347	345	CUAUAGAAAUAAGUGAUGUdTdT	ACAUCACUUAUUUCUAUAGdTdT	AL-DP-2031	15				
554	552	UCAAAACAACACUCUUGAAdTdT	UUCAAGAGUGUUGUUUUGAdTdT	AL-DP-2122					
1004	1002	UAGAGGGAUUUAUUAUGUCdTdT	GACAUAAUAAAUCCCUCUAdTdT	AL-DP-2123					
1408	1406	AUAAAAGGGUUUGUAAAUAdTdT	UAUUUACAAACCCUUUUAUdTdT	AL-DP-2124					
1867	1865	CUCAGUGUAGGUAGAAUGUdTdT	ACAUUCUACCUACACUGAGdTdT	AL-DP-2032	90				
1868	1866	UCAGUGUAGGUAGAAUGUUdTdT	AACAUUCUACCUACACUGAdTdT	AL-DP-2033	84				
1869	1867	CAGUGUAGGUAGAAUGUUUdTdT	AAACAUUCUACCUACACUGdTdT	AL-DP-2034	86				
1870	1868	AGUGUAGGUAGAAUGUUUGdTdT	CAAACAUUCUACCUACACUdTdT	AL-DP-2112					
1871	1869	GUGUAGGUAGAAUGUUUGCdTdT	GCAAACAUUCUACCUACACdTdT	AL-DP-2113					
1978	1976	ACAAGAUAUGGUGAUCUAGdTdT	CUAGAUCACCAUAUCUUGUdTdT	AL-DP-2035	89				
2104	2102	AGCAAAUUCAAUCAAGCAUdTdT	AUGCUUGAUUGAAUUUGCUdTdT	AL-DP-2036	87				

2105	2103	GCAAAUUCAAUCAAGCAUUdTdT	AAUGCUUGAUUGAAUUUGCdTdT	AL-DP-2037	91				
2290	2288	GAUGAACAAAGUGGAUUAUdTdT	AUAAUCCACUUUGUUCAUCdTdT	AL-DP-2038	11				
2384	2382	UAAUAUCUCUCAAAGGGAAdTdT	UUCCCUUUGAGAGAUAUUAdTdT	AL-DP-2125					
2386	2384	AUAUCUCUCAAAGGGAAAUdTdT	AUUUCCCUUUGAGAGAUAUdTdT	AL-DP-2126					
2387	2385	UAUCUCUCAAAGGGAAAUUdTdT	AAUUUCCCUUUGAGAGAUAdTdT	AL-DP-2127					
2485	2483	CAUGCUCAAGCAGAUUAUUdTdT	AAUAAUCUGCUUGAGCAUGdTdT	AL-DP-2039	87				
2487	2485	UGCUCAAGCAGAUUAUUUGdTdT	CAAAUAAUCUGCUUGAGCAdTdT	AL-DP-2040	88				
2507	2505	UAGCAUUAAAUAGCCUUAAdTdT	UUAAGGCUAUUUAAUGCUAdTdT	AL-DP-2041	96	76	73	69	94
2508	2506	AGCAUUAAAUAGCCUUAAAdTdT	UUUAAGGCUAUUUAAUGCUdTdT	AL-DP-2114					
2509	2507	GCAUUAAAUAGCCUUAAAUdTdT	AUUUAAGGCUAUUUAAUGCdTdT	AL-DP-2042	96	98	97	97	90
2510	2508	CAUUAAAUAGCCUUAAAUUdTdT	AAUUUAAGGCUAUUUAAUGdTdT	AL-DP-2043	97	86	79	75	94
2765	2763	UAUUAUGCAGUUUAAUAUUdTdT	AAUAUUAAACUGCAUAAUAdTdT	AL-DP-2044	97	79	72	67	84
2767	2765	UUAUGCAGUUUAAUAUUUAdTdT	UAAAUAUUAAACUGCAUAAdTdT	AL-DP-2045	15				
3283	3281	AAAAGUGCACAACAUUAUAdTdT	UAUAAUGUUGUGCACUUUUdTdT	AL-DP-2128					
3284	3282	AAAGUGCACAACAUUAUACdTdT	GUAUAAUGUUGUGCACUUUdTdT	AL-DP-2046	94	94	91	91	93
3338	3336	AUAUAGAACCUACAUAUCCdTdT	GGAUAUGUAGGUUCUAUAUdTdT	AL-DP-2047	87				
3339	3337	UAUAGAACCUACAUAUCCUdTdT	AGGAUAUGUAGGUUCUAUAdTdT	AL-DP-2048	84				
3365	3363	UAAGAGUUGUUUAUGAAAGdTdT	CUUUCAUAAACAACUCUUAdTdT	AL-DP-2129					
4021	4019	ACAGUCAGUAGUAGACCAUdTdT	AUGGUCUACUACUGACUGUdTdT	AL-DP-2049	24				
4022	4020	CAGUCAGUAGUAGACCAUGdTdT	CAUGGUCUACUACUGACUGdTdT	AL-DP-2050	15				
4023	4021	AGUCAGUAGUAGACCAUGUdTdT	ACAUGGUCUACUACUGACUdTdT	AL-DP-2051	87				
4024	4022	GUCAGUAGUAGACCAUGUGdTdT	CACAUGGUCUACUACUGACdTdT	AL-DP-2052	96	84	76	69	87
4025	4023	UCAGUAGUAGACCAUGUGAdTdT	UCACAUGGUCUACUACUGAdTdT	AL-DP-2053	92	84	79	76	74
4037	4035	CAUGUGAAUUCCCUGCAUCdTdT	GAUGCAGGGAAUUCACAUGdTdT	AL-DP-2054	97	79	78	69	96
4038	4036	AUGUGAAUUCCCUGCAUCAdTdT	UGAUGCAGGGAAUUCACAUdTdT	AL-DP-2055	88				
4039	4037	UGUGAAUUCCCUGCAUCAAdTdT	UUGAUGCAGGGAAUUCACAdTdT	AL-DP-2056	16				
4040	4038	GUGAAUUCCCUGCAUCAAUdTdT	AUUGAUGCAGGGAAUUCACdTdT	AL-DP-2115					
4043	4041	AAUUCCCUGCAUCAAUACCdTdT	GGUAUUGAUGCAGGGAAUUdTdT	AL-DP-2057	94	91	86	79	69
4051	4049	GCAUCAAUACCAGCUUAUAdTdT	UAUAAGCUGGUAUUGAUGCdTdT	AL-DP-2058	86				
4052	4050	CAUCAAUACCAGCUUAUAGdTdT	CUAUAAGCUGGUAUUGAUGdTdT	AL-DP-2059	91				
4057	4055	AUACCAGCUUAUAGAACAAdTdT	UUGUUCUAUAAGCUGGUAUdTdT	AL-DP-2060	92				
4058	4056	UACCAGCUUAUAGAACAACdTdT	GUUGUUCUAUAAGCUGGUAdTdT	AL-DP-2061	88				

4059	4057	ACCAGCUUAUAGAACAACAdTdT	UGUUGUUCUAUAAGCUGGUdTdT	AL-DP-2062	95	79	78	72	94
4060	4058	CCAGCUUAUAGAACAACAAdTdT	UUGUUGUUCUAUAAGCUGGdTdT	AL-DP-2063	90				
4061	4059	CAGCUUAUAGAACAACAAAdTdT	UUUGUUGUUCUAUAAGCUGdTdT	AL-DP-2064	94	86	76	67	83
4067	4065	AUAGAACAACAAAUUAUCAdTdT	UGAUAAUUUGUUGUUCUAUdTdT	AL-DP-2065	91				
4112	4110	UAUUAACAGAAAAGUAUGGdTdT	CCAUACUUUUCUGUUAAUAdTdT	AL-DP-2130					
4251	4249	UGAGAUACAUUUGAUGAAAdTdT	UUUCAUCAAAUGUAUCUCAdTdT	AL-DP-2066	86				
4252	4250	GAGAUACAUUUGAUGAAACdTdT	GUUUCAUCAAAUGUAUCUCdTdT	AL-DP-2067	92				
4254	4252	GAUACAUUUGAUGAAACCUdTdT	AGGUUUCAUCAAAUGUAUCdTdT	AL-DP-2068	93				
4255	4253	AUACAUUUGAUGAAACCUCdTdT	GAGGUUUCAUCAAAUGUAUdTdT	AL-DP-2069	89				
4256	4254	UACAUUUGAUGAAACCUCCdTdT	GGAGGUUUCAUCAAAUGUAdTdT	AL-DP-2074					
4313	4311	AAGUGAUACAAAAACAGCAdTdT	UGCUGUUUUUGUAUCACUUdTdT	AL-DP-2131					
4314	4312	AGUGAUACAAAAACAGCAUdTdT	AUGCUGUUUUUGUAUCACUdTdT	AL-DP-2132					
4316	4314	UGAUACAAAAACAGCAUAUdTdT	AUAUGCUGUUUUUGUAUCAdTdT	AL-DP-2133					
4473	4471	UUUAAGUACUAAUUUAGCUdTdT	AGCUAAAUUAGUACUUAAAdTdT	AL-DP-2075					
4474	4472	UUAAGUACUAAUUUAGCUGdTdT	CAGCUAAAUUAGUACUUAAdTdT	AL-DP-2076					
4475	4473	UAAGUACUAAUUUAGCUGGdTdT	CCAGCUAAAUUAGUACUUAdTdT	AL-DP-2077					
4476	4474	AAGUACUAAUUUAGCUGGAdTdT	UCCAGCUAAAUUAGUACUUdTdT	AL-DP-2078					
4477	4475	AGUACUAAUUUAGCUGGACdTdT	GUCCAGCUAAAUUAGUACUdTdT	AL-DP-2079					
4478	4476	GUACUAAUUUAGCUGGACAdTdT	UGUCCAGCUAAAUUAGUACdTdT	AL-DP-2080					
4480	4478	ACUAAUUUAGCUGGACAUUdTdT	AAUGUCCAGCUAAAUUAGUdTdT	AL-DP-2081					
4483	4481	AAUUUAGCUGGACAUUGGAdTdT	UCCAAUGUCCAGCUAAAUUdTdT	AL-DP-2082					
4484	4482	AUUUAGCUGGACAUUGGAUdTdT	AUCCAAUGUCCAGCUAAAUdTdT	AL-DP-2083					
4486	4484	UUAGCUGGACAUUGGAUUCdTdT	GAAUCCAAUGUCCAGCUAAdTdT	AL-DP-2084					
4539	4537	UUUUGAAAAAGAUUGGGGAdTdT	UCCCCAAUCUUUUUCAAAAdTdT	AL-DP-2134					
4540	4538	UUUGAAAAAGAUUGGGGAGdTdT	CUCCCCAAUCUUUUUCAAAdTdT	AL-DP-2135					
4542	4540	UGAAAAAGAUUGGGGAGAGdTdT	CUCUCCCCAAUCUUUUUCAdTdT	AL-DP-2136					
4543	4541	GAAAAAGAUUGGGGAGAGGdTdT	CCUCUCCCCAAUCUUUUUCdTdT	AL-DP-2137					
4671	4669	UAUGAACACUUCAGAUCUUdTdT	AAGAUCUGAAGUGUUCAUAdTdT	AL-DP-2085					
4672	4670	AUGAACACUUCAGAUCUUCdTdT	GAAGAUCUGAAGUGUUCAUdTdT	AL-DP-2086					
4867	4865	UGCCCUUGGGUUGUUAACAdTdT	UGUUAACAACCCAAGGGCAdTdT	AL-DP-2087					
4868	4866	GCCCUUGGGUUGUUAACAUdTdT	AUGUUAACAACCCAAGGGCdTdT	AL-DP-2088					
5544	5542	UAUAGCAUUCAUAGGUGAAdTdT	UUCACCUAUGAAUGCUAUAdTdT	AL-DP-2089					
5545	5543	AUAGCAUUCAUAGGUGAAGdTdT	CUUCACCUAUGAAUGCUAUdTdT	AL-DP-2090					
5546	5544	UAGCAUUCAUAGGUGAAGGdTdT	CCUUCACCUAUGAAUGCUAdTdT	AL-DP-2091					
5550	5548	AUUCAUAGGUGAAGGAGCAdTdT	UGCUCCUUCACCUAUGAAUdTdT	AL-DP-2092					

5640	5638	UUGCAAUGAUCAUAGUUUAdTdT	UAAACUAUGAUCAUUGCAAdTdT	AL-DP-2093
5641	5639	UGCAAUGAUCAUAGUUUACdTdT	GUAAACUAUGAUCAUUGCAdTdT	AL-DP-2094
5642	5640	GCAAUGAUCAUAGUUUACCdTdT	GGUAAACUAUGAUCAUUGCdTdT	AL-DP-2095
5643	5641	CAAUGAUCAUAGUUUACCUdTdT	AGGUAAACUAUGAUCAUUGdTdT	AL-DP-2096
5644	5642	AAUGAUCAUAGUUUACCUAdTdT	UAGGUAAACUAUGAUCAUUdTdT	AL-DP-2097
5645	5643	AUGAUCAUAGUUUACCUAUdTdT	AUAGGUAAACUAUGAUCAUdTdT	AL-DP-2098
5647	5645	GAUCAUAGUUUACCUAUUGdTdT	CAAUAGGUAAACUAUGAUCdTdT	AL-DP-2138
5648	5646	AUCAUAGUUUACCUAUUGAdTdT	UCAAUAGGUAAACUAUGAUdTdT	AL-DP-2139
5649	5647	UCAUAGUUUACCUAUUGAGdTdT	CUCAAUAGGUAAACUAUGAdTdT	AL-DP-2140
5650	5648	CAUAGUUUACCUAUUGAGUdTdT	ACUCAAUAGGUAAACUAUGdTdT	AL-DP-2099
5651	5649	AUAGUUUACCUAUUGAGUUdTdT	AACUCAAUAGGUAAACUAUdTdT	AL-DP-2100
5752	5750	CAUUGGUCUUAUUUACAUAdTdT	UAUGUAAAUAAGACCAAUGdTdT	AL-DP-2101
5754	5752	UUGGUCUUAUUUACAUAUAdTdT	UAUAUGUAAAUAAGACCAAdTdT	AL-DP-2102
5755	5753	UGGUCUUAUUUACAUAUAAdTdT	UUAUAUGUAAAUAAGACCAdTdT	AL-DP-2103
5756	5754	GGUCUUAUUUACAUAUAAAdTdT	UUUAUAUGUAAAUAAGACCdTdT	AL-DP-2141
5919	5917	AUAUCAUGCUCAAGAUGAUdTdT	AUCAUCUUGAGCAUGAUAUdTdT	AL-DP-2142
5920	5918	UAUCAUGCUCAAGAUGAUAdTdT	UAUCAUCUUGAGCAUGAUAdTdT	AL-DP-2104
5934	5932	UGAUAUUGAUUUCAAAUUAdTdT	UAAUUUGAAAUCAAUAUCAdTdT	AL-DP-2105
6016	6014	UACUUAGUCCUUACAAUAGdTdT	CUAUUGUAAGGACUAAGUAdTdT	AL-DP-2106
6019	6017	UUAGUCCUUACAAUAGGUCdTdT	GACCUAUUGUAAGGACUAAdTdT	AL-DP-2107
6020	6018	UAGUCCUUACAAUAGGUCCdTdT	GGACCUAUUGUAAGGACUAdTdT	AL-DP-2108
6252	6250	AUAUUCUAUAGCUGGACGUdTdT	ACGUCCAGCUAUAGAAUAUdTdT	AL-DP-2109
6253	6251	UAUUCUAUAGCUGGACGUAdTdT	UACGUCCAGCUAUAGAAUAdTdT	AL-DP-2110
6254	6252	AUUCUAUAGCUGGACGUAAdTdT	UUACGUCCAGCUAUAGAAUdTdT	AL-DP-2111

Table 1b. RSV P gene

Actual start	Start_Pos	Sense	Antisense	AL-DP #	% Inhibition (5nM)	% Inhibition RSV A2 500 pM	% Inhibition RSV A2 50 pM	% Inhibition RSV A2 5pM	% Inhibition RSV B (5nM)
55	53	AAAUUCCUAGAAUCAAUAAdTdT	UUAUUGAUUCUAGGAAUUUdTdT	AL-DP-2000	3				
56	54	AAUUCCUAGAAUCAAUAAAdTdT	UUUAUUGAUUCUAGGAAUUdTdT	AL-DP-2001	4				
58	56	UUCCUAGAAUCAAUAAAGGdTdT	CCUUUAUUGAUUCUAGGAAdTdT	AL-DP-2002	7				
59	57	UCCUAGAAUCAAUAAAGGGdTdT	CCCUUUAUUGAUUCUAGGAdTdT	AL-DP-2003	98	93	92	84	97
81	59	CUAGAAUCAAUAAAGGGCAdTdT	UGCCCUUUAUUGAUUCUAGdTdT	AL-DP-2004	3				
322	320	ACAUUUGAUAACAAUGAAGdTdT	CUUCAUUGUUAUCAAAUGUdTdT	AL-DP-2005	7				
323	321	CAUUUGAUAACAAUGAAGAdTdT	UCUUCAUUGUUAUCAAAUGdTdT	AL-DP-2006	5				
324	322	AUUUGAUAACAAUGAAGAAdTdT	UUCUUCAUUGUUAUCAAAUdTdT	AL-DP-2007	4				
325	323	UUUGAUAACAAUGAAGAAGdTdT	CUUCUUCAUUGUUAUCAAAdTdT	AL-DP-2008	7				
426	424	AAGUGAAAUACUAGGAAUGdTdT	CAUUCCUAGUAUUUCACUUdTdT	AL-DP-2009	2				
427	425	AGUGAAAUACUAGGAAUGCdTdT	GCAUUCCUAGUAUUUCACUdTdT	AL-DP-2010	7				
428	426	GUGAAAUACUAGGAAUGCUdTdT	AGCAUUCCUAGUAUUUCACdTdT	AL-DP-2011	4				
429	427	UGAAAUACUAGGAAUGCUUdTdT	AAGCAUUCCUAGUAUUUCAdTdT	AL-DP-2012	96	77	68	66	92
430	428	GAAAUACUAGGAAUGCUUCdTdT	GAAGCAUUCCUAGUAUUUCdTdT	AL-DP-2013	98	85	76	75	89
431	429	AAAUACUAGGAAUGCUUCAdTdT	UGAAGCAUUCCUAGUAUUUdTdT	AL-DP-2014	98	85	81	68	66
550	548	GAAGCAUUAAUGACCAAUGdTdT	CAUUGGUCAUUAAUGCUUCdTdT	AL-DP-2015	7				
551	549	AAGCAUUAAUGACCAAUGAdTdT	UCAUUGGUCAUUAAUGCUUdTdT	AL-DP-2016	98	88	82	75	94
		CGAUAAUAUAACAGCAAGAdTsdT	UCUUGCUGUUAUAUUAUCGdTsdT	AL-DP-1729	90				
		CGAUUAUAUUACAGGAUGAdTsdT	UCAUCCUGUAAUAUAAUCGdTsdT	AL-DP-1730					

Table 1c. RSV N gene

Actual start	Sense	Antisense	AL- DP #	% inhibition (5nM)	% inhibition RSV A2 500 pM	% inhibition RSV A2 50 pM	% inhibition RSV A2 5pM	% inhibition RSV B (5nM)
3	GGCUCUUAGCAAAGUCAAGdTdT	CUUGACUUUGCUAAGAGCCdTdT	AL-DP-2017	98	86	84	80	93
5	CUCUUAGCAAAGUCAAGUUdTdT	AACUUGACUUUGCUAAGAGdTdT	AL-DP-2018	2				
52	CUGUCAUCCAGCAAAUACAdTdT	UGUAUUUGCUGGAUGACAGdTdT	AL-DP-2019	5				
53	UGUCAUCCAGCAAAUACACdTdT	GUGUAUUUGCUGGAUGACAdTdT	AL-DP-2020	2				
191	UAAUAGGUAUGUUAUAUGCdTdT	GCAUAUAACAUACCUAUUAdTdT	AL-DP-2021	3				
379	AUUGAGAUAGAAUCUAGAAdTdT	UUCUAGAUUCUAUCUCAAUdTdT	AL-DP-2022	98	78	77	75	94
897	AUUCUACCAUAUAUUGAACdTdT	GUUCAAUAUAUGGUAGAAUdTdT	AL-DP-2023	1				
898	UUCUACCAUAUAUUGAACAdTdT	UGUUCAAUAUAUGGUAGAAdTdT	AL-DP-2024	7				
899	UCUACCAUAUAUUGAACAAdTdT	UUGUUCAAUAUAUGGUAGAdTdT	AL-DP-2025	96	89	84	77	96

WHAT IS CLAIMED IS:

1. A method of reducing the levels of a viral protein, viral mRNA or viral titer in a cell in a subject comprising the step of administering an iRNA agent to said subject, wherein the iRNA agent comprising a sense strand having at least 15 or more contiguous nucleotides complementary to gene from a first mammalian respiratory virus and an antisense strand having at least 15 or more contiguous nucleotides complementary to said sense strand, wherein said gene is selected from the group consisting of the P N or L gene of RSV.

2. The method of claim 1 wherein said agent comprises 15 or more nucleotides selected from one of the agents of Table 1 (a-c).

3. The method of claim 1, wherein said the iRNA agent is administered intranasally to a subject.

4. The method of claim 1, wherein said the iRNA agent is administered via inhalation or nebulization to a subject.

5. The method of claim 1, wherein said the iRNA agent reduces the viral titer in said subject.

6. The method of claim 1 further comprising co-administering a second iRNA agent to said subject, wherein said second iRNA agent comprising a sense strand having at least 15 or more contiguous nucleotides complementary to second gene from said respiratory virus and an antisense strand having at least 15 or more contiguous nucleotides complementary to said sense strand.

7. The method of claim 6 wherein said agent comprises 15 or more nucleotides selected from one of the agents of Table 1 (a-c).

8. The method of claim 6, wherein the subject is diagnosed as having a viral infection with said first and said second mammalian respiratory virus.

9. A method of reducing the levels of a viral protein from a first and a second gene of a respiratory virus in a cell in a subject comprising the step of co-administering a first and a second iRNA agent to said subject, wherein said first iRNA agent comprising a sense strand having at least 15 or more contiguous nucleotides complementary to a first gene from a mammalian respiratory virus and an antisense strand having at least 15 or more contiguous nucleotides complementary to said sense strand and said second iRNA agent comprising a sense strand having at least 15 or more contiguous nucleotides complementary to a second gene from said mammalian respiratory virus and an antisense strand having at least 15 or more contiguous nucleotides complementary to said sense strand.

10. The method of claim 9 wherein said agents comprises 15 or more nucleotides selected from one of the agents of Table 1 (a-c).

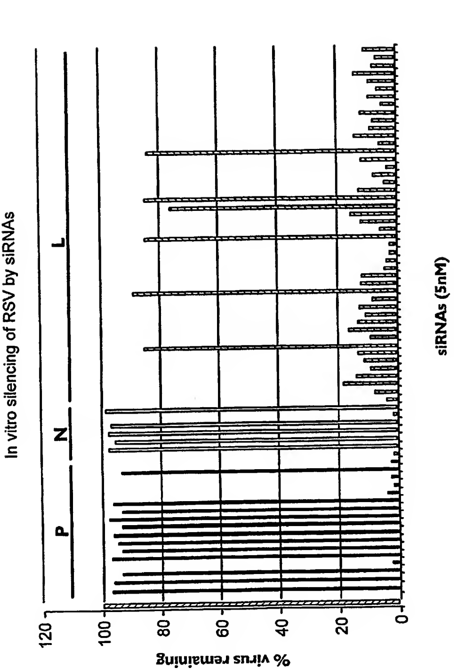
In vitro silencing of RSV by siRNAs
P
N
L
120
100
80
60
40
20
0
% virus remaining
siRNAs (5nM)

FIG. 1

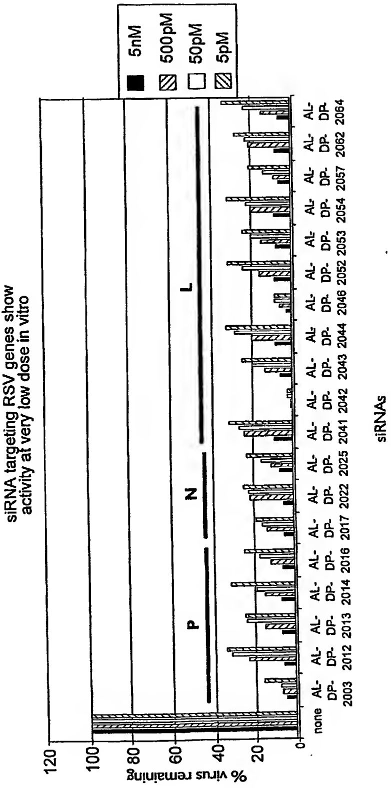
siRNA targeting RSV genes show activity at very low dose in vitro
% virus remaining
120
100
80
60
40
20
0
5nM
500pM
50pM
5pM
P
N
L
none
AL-DP-2003
AL-DP-2012
AL-DP-2013
AL-DP-2014
AL-DP-2016
AL-DP-2017
AL-DP-2022
AL-DP-2025
AL-DP-2041
AL-DP-2042
AL-DP-2043
AL-DP-2044
AL-DP-2046
AL-DP-2052
AL-DP-2053
AL-DP-2054
AL-DP-2057
AL-DP-2062
AL-DP-2064
siRNAs

FIG. 2

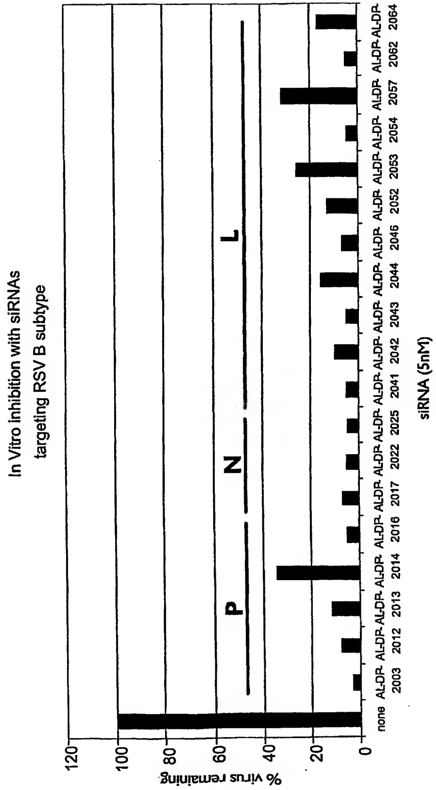
In Vitro inhibition with siRNAs
targeting RSV B subtype
% virus remaining
120
100
80
60
40
20
0
P
N
L
none
AL-DP-2003
AL-DP-2012
AL-DP-2013
AL-DP-2014
AL-DP-2016
AL-DP-2017
AL-DP-2022
AL-DP-2025
AL-DP-2041
AL-DP-2042
AL-DP-2043
AL-DP-2044
AL-DP-2046
AL-DP-2052
AL-DP-2053
AL-DP-2054
AL-DP-2057
AL-DP-2062
AL-DP-2064
siRNA (5nM)

FIG. 3

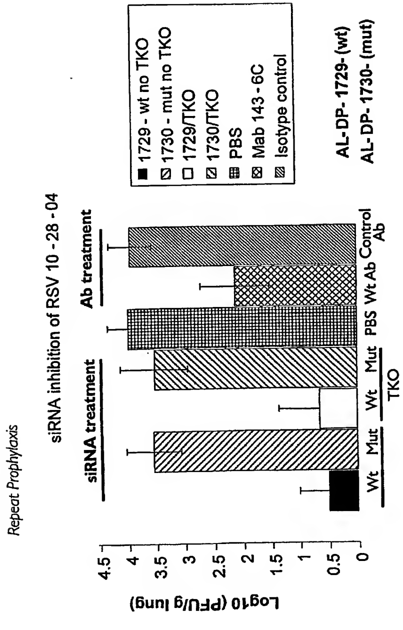
Repeat Prophylaxis
siRNA inhibition of RSV 10 - 28 - 04
siRNA treatment
Ab treatment
Log10 (PFU/g lung)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
Wt
Mut
Wt
Mut
TKO
PBS
Wt Ab
Control Ab
1729 - wt no TKO
1730 - mut no TKO
1729/TKO
1730/TKO
PBS
Mab 143 - 6C
Isotype control
AL-DP- 1729- (wt)
AL-DP- 1730- (mut)

FIG. 4

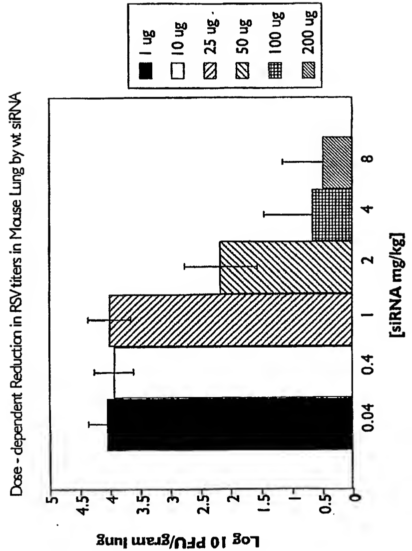
Dose - dependent Reduction in RSV titers in Mouse Lung by wt. siRNA
Log 10 PFU/gram lung
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
5
0.04
0.4
1
2
4
8
[siRNA mg/kg]
1 ug
10 ug
25 ug
50 ug
100 ug
200 ug

FIG. 5

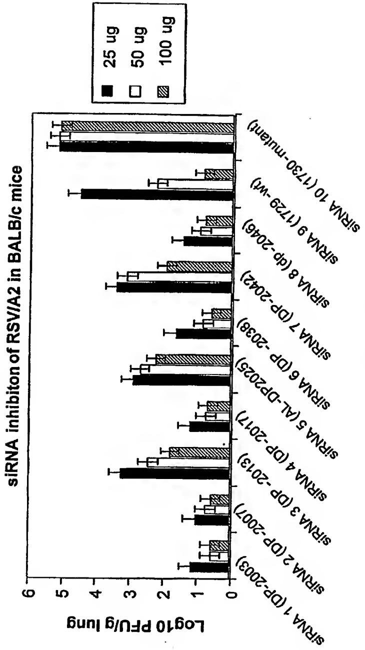
siRNA inhibiton of RSV/A2 in BALB/c mice
Log10 PFU/g lung
0
1
2
3
4
5
6
25 ug
50 ug
100 ug
siRNA 1 (DP-2003)
siRNA 2 (DP-2007)
siRNA 3 (DP-2013)
siRNA 4 (DP-2017)
siRNA 5 (AL-DP2025)
siRNA 6 (DP-2038)
siRNA 7 (DP-2042)
siRNA 8 (dp-2046)
siRNA 9 (1729-wt)
siRNA 10 (1730-mutant)

FIG. 6

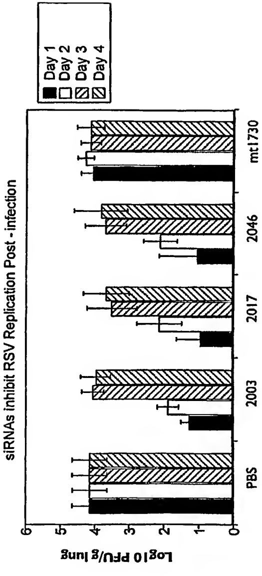
siRNAs inhibit RSV Replication Post - infection
Log10 PFU/g lung
0
1
2
3
4
5
6
PBS
2003
2017
2046
mt1730
Day 1
Day 2
Day 3
Day 4

FIG. 7

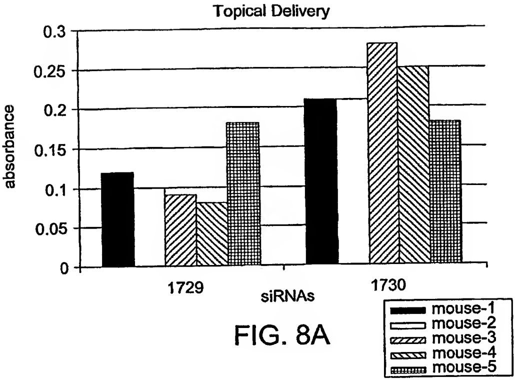
Topical Delivery
0.3
0.25
0.2
0.15
0.1
0.05
0
absorbance
1729
1730
siRNAs
mouse-1
mouse-2
mouse-3
mouse-4
mouse-5

FIG. 8A

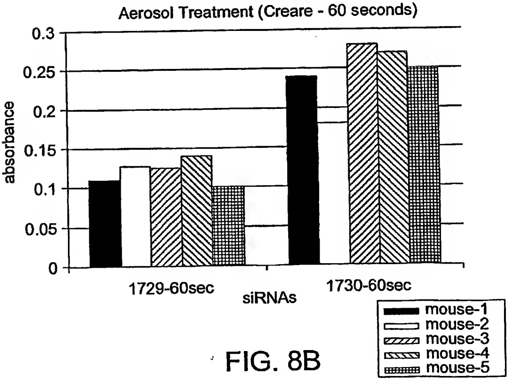
Aerosol Treatment (Creare - 60 seconds)
0.3
0.25
0.2
0.15
0.1
0.05
0
absorbance
1729-60sec
1730-60sec
siRNAs
mouse-1
mouse-2
mouse-3
mouse-4
mouse-5

FIG. 8B

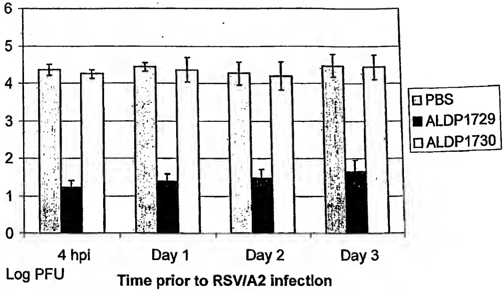
6
5
4
3
2
1
0
4 hpi
Day 1
Day 2
Day 3
Log PFU
Time prior to RSV/A2 infection
PBS
ALDP1729
ALDP1730

FIG. 9